



PHD

An investigation into CCR4 T lymphocyte signalling and chemotaxis

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AN INVESTIGATION INTO CCR4 T LYMPHOCYTE SIGNALLING AND CHEMOTAXIS

Submitted by

DARRAN G. CRONSHAW

For the degree of PhD
of the University of Bath
2004

A handwritten signature in black ink, appearing to read 'D. Cronshaw', is written diagonally across the left side of the page.

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PhD.

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One thing that is apparent to me now is that....

"Being a graduate student is like becoming all of the Seven Dwarves. In the beginning you're Dopey and Bashful. In the middle, you are usually sick (Sneezy), tired (Sleepy), and irritable (Grumpy). But at the end, they call you Doc, and then you're Happy." - R. T. Azuma.

And then you realise...

"Science is a wonderful thing if one does not have to earn one's living at it." - A. Einstein.

Nevermind.

Abstract

The research presented within this thesis concentrates on the signal transduction pathways downstream of the chemokine receptor CCR4, and those pathways that are critical for T cell migration. Chemokines are a family of small molecular weight (~ 8 – 11 kDa) proteins that have been divided into 4 groups based on their arrangement of 4 highly conserved amino-terminal cysteine residues. Chemokines are critical factors involved in the localisation of leukocytes (and other cell types) to various areas within the body for differentiation/maturation and immunity processes. However, chemokines have been implicated in the dysregulation of the immune process due to an over exuberant/unnecessary recruitment of cells to areas of antigen and this leads to a debilitating inflammatory/autoimmune response. Thus, a greater understanding of the biochemical and functional pathways mediated by CCR4 may potentially provide novel therapeutic targets. Until now, CCR4 signalling events have been poorly characterised.

Results presented within demonstrate the ability of MDC/TARC in a dose-dependent and pertussis toxin *sensitive* manner to flux calcium in a leukaemic T cell line (CEM) and polarised human Th2 cells. Pre-incubation of these cells with the PLC inhibitor, U73122, resulted in the abrogation of CCR4-mediated calcium mobilisation. This MDC-induced calcium response was found to be wholly *sensitive* to the IP₃ receptor antagonist 2-APB. MDC is able to induce the pertussis toxin *sensitive* migration of CEM and polarised human Th2 cells. Th2/CEM cell migration in response to MDC is *sensitive* to the PLC and PKC selective inhibitors U73122 and RO-32-0432. However, 2-APB has no effect, implicating a role for a novel PKC isoform (Ca^{2+} -*independent*, DAG-*dependent*). This observation is supported by the ability of Rottlerin (PKC δ inhibitor) to abrogate this MDC-induced T cell chemotaxis. Surprisingly, this CCR4-mediated chemotaxis is *insensitive* to the PI3K inhibitors, LY294002 (to 30 μM) and wortmannin (to 300nM). Yet, PI3K is activated downstream of CCR4 as demonstrated by MDC-induced PIP3 accumulation, and activation of the PI3K isoforms, p110 δ and p110 γ (each being Wortmannin/LY294002 sensitive). Additionally, studies have demonstrated that this MDC-mediated CEM cell chemotaxis is *sensitive* to the ROCK inhibitor, Y27632, with MDC possessing the ability to phosphorylate the downstream ROCK effector MLC.

These results indicate that whilst CCR4 activates analogous pathways to other chemokine receptors, there are distinct dissimilarities in the cascades that are required for directed cell migration between the different cell and chemokine/receptor types.

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Abbreviations

Signalling Pathways

APC	antigen presenting cell
ARF	ADP ribosylating factor
Arno	ARF nucleotide-binding site opener
Btk	Bruton's tyrosine kinase
Cam	calmodulin
CaMK	calcium/calmodulin-dependent kinase
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CD	cluster of differentiation
Cdc42	cell division cycle protein 42, a G-protein
CDK	cyclin-dependent kinase
CREB	cAMP response element-binding protein
DAG	diacylglycerol
dsRNA	double-stranded RNA
ELK-1	Ets domain protein
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase (MAPK)
FAK	focal adhesion kinase
FKHR	forkhead in Rhabdomyosarcoma
fMLP	formyl-methionyl-leucyl-phenylalanine
Gab1	GRB2-associated binder-1
GAP	GTPase activating protein
GEF	guanine nucleotide exchange factor
GPCR	G-protein coupled receptor
GRB2	growth factor receptor-bound protein-2
GRK	G-protein coupled receptor kinase
GSK-3	glycogen synthase kinase-3
I κ B	inhibitor of NF- κ B
IKK	I κ B kinase
IP3R	inositol 1,4,5-trisphosphate receptor
IP3	inositol 1,4,5-trisphosphate
JAK	Janus-family tyrosine kinase
JNK	Jun N-terminal kinase
KSR	kinase suppressor of Ras
MAPK	mitogen-activated protein kinase
MAPKAP-2	MAP kinase-activated protein kinase 2
MEK	MAPK/ERK kinase (MAPKK)
MEKK	MEK kinase
MKK	Sek or JNK kinase
MKP	MAP kinase phosphatase
MLC2	myosin light chain 2
MLCK	myosin light chain kinase
MSK-1	mitogen and stress-activated kinase 1
mTOR	mammalian target of rapamycin

NFAT	nuclear factor of activated T cells
NF- κ B	nuclear factor kappa B
p90RSK	90 kDa ribosomal S6 kinase
PAK	p21-activated protein kinase
PDE	phosphodiesterase
PDK	3-phosphoinositide-dependent protein kinase
PH	pleckstrin homology domain
PI3K	phosphoinositide-3-kinase
PIP2/PI(4,5)P ₂	phosphatidylinositol 3,4-bisphosphate
PIP3/PI(3,4,5)P ₃	phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A (cAMP-dependent protein kinase)
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLP2A	phospholipase 2A
PP2A	phosphoprotein phosphatase 2A
PPAR γ	peroxisome proliferator-activated receptor
PTB	phosphotyrosine binding domain
PTEN	phosphatase and tensin homologue deleted on chromosome ten
PYK2	proline-rich tyrosine kinase
RGS	regulator of G protein signalling
ROCK	rho-associated, coiled-coil-containing protein kinase
RSK	ribosomal S6 kinase
RTK	receptor tyrosine kinase
RR	ryanodine receptors
S6	small subunit ribosomal protein S6
S6K	S6 kinase
SAPK	stress-activated protein kinase
SEK	SAPK kinase
SH2	Src-homology 2 domain
SH3	Src-homology 3 domain
Shc	SH2-containing collagen-related proteins (adaptors)
SHP	SH2-containing phosphatase
Sos	son of sevenless guanine nucleotide exchange factor
SRE	serum response element
SRF	serum response factor
STAT	signal transducer and activator of transcription

Chemokine Acronyms

6Ckine	chemokine with 6 cysteines
AMAC	alternative macrophage activation-associated CC chemokine
ATAC	activation-induced, chemokine-related molecule exclusively expressed in CD8 ⁺ T lymphocytes.
BCA-1	B cell-activating chemokine-1
BLC	B lymphocyte Chemoattractant
BRAK	breast and kidney chemokine

CTACK	cutaneous T cell-attracting chemokine
DARC	Duffy antigen receptor for chemokines
DC-CK-1	dendritic cell chemokine 1
ELC	Epstein-Barr virus-induced receptor ligand chemokine
ENA-78	epithelial cell-derived neutrophil-activating factor, 78 amino acids
GCP	granulocyte chemoattractant protein
Gro	growth-related oncogene
HCC	haemofiltrate CC chemokine
IL-8	interleukin-8
IP-10	interferon- γ -inducible protein-10
ITAC	interferon-inducible T cell α chemoattractant
LARC	liver- and activation-related chemokine
LEC	liver-expressed chemokine
Lkn-1	leukotactin-1
MCP	monocyte chemoattractant protein
MDC	macrophage-derived chemokine
MEC	mucosae-associated epithelial chemokine
MGSA	melanoma growth-stimulatory activity
Mig	monokine induced by γ -interferon
MIP	macrophage inflammatory protein
MPIF	myeloid progenitor inhibitory factor
NAP	neutrophil-activating protein
PARC	pulmonary- and activation-regulated chemokine
PF-4	platelet factor-4
RANTES	regulated on activation, normal T cell expressed and secreted
SDF-1	stromal cell-derived factor-1
SLC	secondary lymphoid tissue chemokine
STCP-1	stimulated T cell chemoattractant protein-1
TARC	thymus- and activation-related chemokine
TECK	thymus-expressed chemokine

Inhibitors

AG490	α -Cyano-(3,4-dihydroxy)-N-benzylcinnamide; tyrphostin B42; 2-cyano-3-(3,4-dihydroxyphenyl)-N-(benzyl)-2-propenamide; 2-cyano-3-(3,4-dihydroxyphenyl)-N-(phenylmethyl)-2-propenamide
AG9	tyrphostin A1; tyrphostin 1; α -cyano-(4-methoxy)cinnamitrile; (4-methoxybenzylidene)malonitrile
2-APB	2-aminoethoxydiphenylborate
8-Br-cADPR	8-bromo-cyclic adenosine diphosphate ribose
Caffeine	1,3,7-trimethylxanthine
Dantrolene	1-([5-(p-nitrophenyl)furfurylidene]amino)hydantoin
L-NAME	N ^G -nitro-L-arginine methyl ester
LY	LY294002; 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride

Nifedipine	1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester
PD	PD98059; 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PKBi (PKB inhibitor)	1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate
PTX	pertussis toxin, source Bordetella pertussis
RO	RO-32-0432; Bisindoylmaleimide XI; 2-(8-[[{dimethylamino}methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl]-3-(1-methylindol-3-yl)maleimide
Rott	rottlerin; mallotoxin; 1-[6-[(3-acetyl-2,4,6-trihydroxy-5-methylphenyl)methyl]-5,7-dihydroxy-2,2-dimethyl-2H-1-benzopyran-8-yl]-3-phenyl-2-propen-1-one
Ryanodine	ryanodol 3-(1H-pyrrole-2-carboxylate)
SB	SB203580; 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole
TLCK	N ^α -tosyl-lys chloromethyl ketone
TPCK	N ^α -tosyl-phe chloromethyl ketone
U73122	1-(6-[[[17β]-3-Methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl)-1H-pyrrole-2,5-dione
U73343	1-(6-[[[17β]-3-Methoxyestra-1,3,5[10]-trien-17-yl]amino]hexyl)-1H-pyrrolidinedione
WHI-P154	4-[(3'-Bromo-4'-hydroxyphenyl)amino]-6,7-dimethoxyquinazoline
Wort.	Wortmannin, source Penicillium funiculosum

Cell Types/Tissue

APC	antigen presenting cell
B	B cell
Ba	basophil
CHO	Chinese hamster ovary cell
CNS	central nervous system
DC	dendritic cell
iDC	immature dendritic cell
mDC	mature dendritic cell
Eo	eosinophil
F	fibroblast
L	leukocyte
LΦ	lymphocyte
M	monocyte
MΦ	macrophage
My	myeloid cell
N/NΦ	neutrophil
NK	natural killer T cell
P	platelet
RBC	red blood cell/erythrocyte

SI	small intestine
SMC	smooth muscle cell
T	T cell
Tc	cytotoxic T cell
Th	helper T cell
Thy	thymocyte
Tn	naïve T cell
Tm	memory T cell

Diseases

A/As	asthma
AD	atopic dermatitis
AS	atherosclerosis
C	cancer
CD	contact dermatitis
ChD	Crohn's disease
CNSi	central nervous system inflammation
D	dermatitis
GN	glomerulonephritis
GVHD	graft-versus-host disease (allograft rejection)
IBD	inflammatory bowel disease
II	intestinal inflammation
LRI	lung reperfusion injury
MS	multiple sclerosis
Ps	psoriasis
RA	rheumatoid arthritis
S	sepsis
SD	sarcoidosis
SKI	skin inflammation
UC	ulcerative colitis

Miscellaneous

Ab	antibody
Abs	absorption maximum (in nanometres)
Acrylamide	N, N'-methylenebisacrylamid
APS	ammonium persulphate
CHCl ₃	chloroform
DMSO	dimethylsulphoxide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N, N, N', N'-tetraacetic acid
Em	fluorescence emission maximum (in nanometres)
EtOH	ethanol
FBS	foetal bovine serum

FITC	fluorescein isothiocyanate
Glycine	aminoacetic acid
Hr	hour(s)
HRP	horseradish peroxidase
Ig	immunoglobulin
MeOH	methanol
Min	minute(s)
MW	molecular weight
NP-40	IGEPAL CA-630
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PMA	phorbol myristate acetate
Probenecid	p-(Dipropylsulfamoyl)benzoic acid
rh	recombinant human
RT	room temperature
SDS	sodium dodecyl sulphate
SEB	staphylococcal enterotoxin B
Sec	second(s)
TBS	tris-buffered saline
TBST	tris-buffered saline with tween-20
TEMED	N, N, N', N'-Tetramethylethylenediamine
TLC	thin layer chromatography
Tris/Trizma® Base	tris(hydroxymethyl)aminomethane
TRITC	tetramethylrhodamine isothiocyanate
Tween-20	polyoxyethylene-sorbitan monolaurate

1 & 3 Letter Amino Acid Code

See 'Appendix 7'.

1

INTRODUCTION

1. INTRODUCTION

1.1 The Immune System

The surrounding environment contains a multitude of pathogens – viruses, bacteria, fungi, protozoa and multicellular parasites – that can cause disease and kill the host if their existence is unchecked. Fortunately, for the human species (and indeed other species), a complex method has evolved over many millennia in which the body is able to distinguish self from what is nonself (i.e. host from foreign) – the IMMUNE SYSTEM. Without the immune system all living matter would be driven into one colossal ball of protoplasm. Thus, organisms have developed this system of molecular recognition that involves cell surface molecules specifically identifying and adhering to molecules on other surfaces that are nonself and eject the offending pathogen from the organism. This immune response can be divided into 2 distinct, but complementary, systems – INNATE and ADAPTIVE immunity (from the latin word *immunis* meaning ‘exempt’). The type of pathogen and the site of infection largely determine the type of immune response.

Innate (non-adaptive) immunity is conferred by all those components present from birth. This form of immunity is always present and available at short notice to prevent the entry of microorganisms or to eject them prior to occurrence of disease, in a non-specific manner. The mechanistic components of the immune system include skin, mucous membranes, coughing and vomiting. Chemical barriers include pH levels (e.g. the acidic environment of the stomach) and antibacterial substances in secretions. Various internal components contribute to the features of the innate immune system, including fever (temperature rise), interferons and some opsonins (e.g. complement) – these affect pathogenic invaders directly or enhance the host’s effectiveness against them. Another component includes the phagocytic cells (monocytes, macrophages, neutrophils) that bind to microorganisms, internalise them and kill them. Phagocytes are able to bind a variety of microbial products making them part of the innate response. The innate response does not alter on repeated exposure to the same pathogen.

Adaptive (acquired) immunity improves with each successive exposure to a given infectious agent and is more specialised than the innate response. Acquired immunity is present exclusively in invertebrates and, in evolutionary terms is a recent phenomenon, with each response specific for a particular immunogen/antigen. This response protects against future invasion by previously encountered pathogens and the primary 'players' in this response are the subset of leukocytes termed 'lymphocytes'. These cells are able to specifically recognise individual pathogens through the expression of antigen-specific receptors (the T cell receptor (TCR) and B cell receptor (BCR) for T and B lymphocytes, respectively). These cells, upon contact with antigen, lead to the synthesis of antibody (B cells – humoral immunity) or cytokines and other soluble factors (T cells – cellular immunity), which help organise a specific response against the presented antigen (fig 1.1).

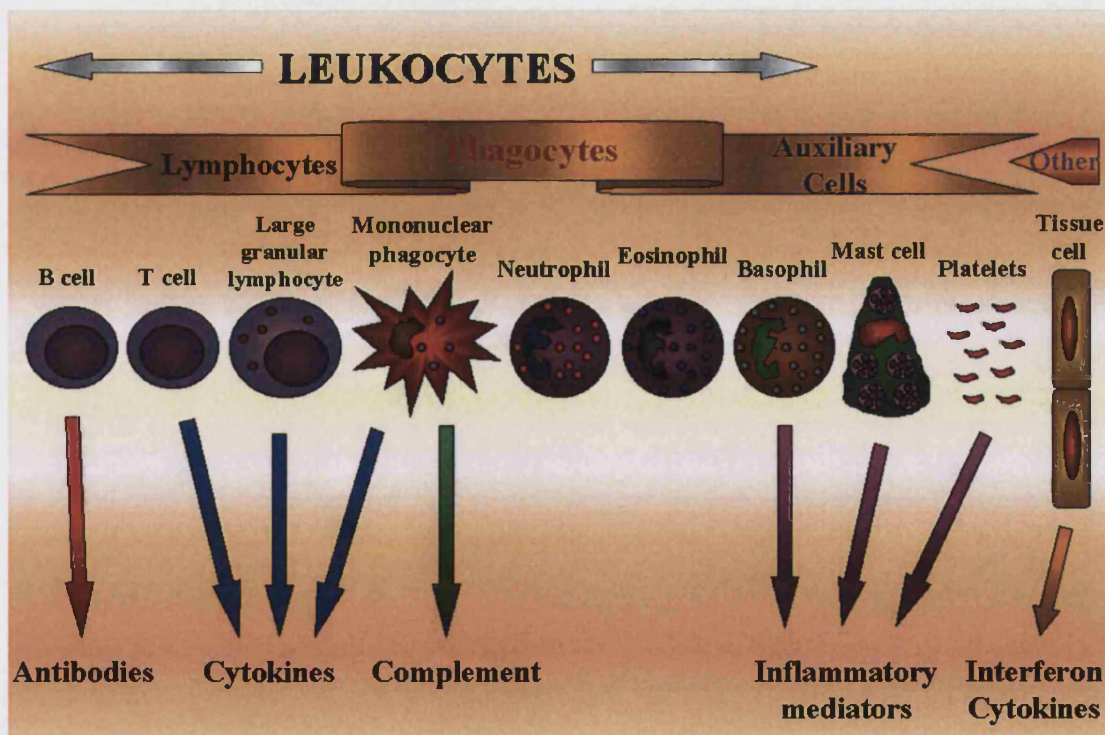


Figure 1.1. Components of the immune system. Figure depicts the principal elements of the immune system, indicating the cells and the soluble mediators that they produce. In addition to the cells displayed, the dendritic cells have a major role in presenting antigen to the lymphocytes (diagram modified from 'Roitt, Brostoff & Male (1998). Immunology, 5th Edition. Pg 3.')

These two arms of the immune response are not mutually exclusive and there is considerable interaction between the two. Some phagocytes, such as macrophages, and other cells (e.g. dendritic cells) are able to present antigen to T lymphocytes in a form they are able to recognise. These cells are known as antigen-presenting cells (APCs) and display two types of molecule that participate in this antigen presentation – MHC (major histocompatibility complex) class I and MHC class II. The processed antigen is non-covalently bound to either of these molecules and presents it to the TCR (MHC I = cytotoxic T cells, MHC II = helper T cells). B cells can act as APCs as well (MHC class II). Additionally, antibody released by B cells is used by phagocytes to more efficiently recognise pathogen, and the soluble proteins released by T cells can recruit more phagocytes to the area of infection.

The cells of the immune system are widely distributed throughout the body, but in the event of invasion by a pathogen it is necessary for the cells (generally leukocytes) of the immune system to be concentrated locally at the area of infection (a process that manifests itself as inflammation). The initial response will require the localisation of cells of the innate system, with APCs such as dendritic cells requiring to migrate from the infected peripheral areas to draining lymph nodes where they present their antigen to T cells. After T cell differentiation and proliferation into antigen-specific T cells, they need to migrate to the periphery. Additionally, if it is a previously encountered antigen then there is a requirement for the mobilisation of memory B and T cells to the area of infection. Besides this mobilisation, leukocytes are generated every day (as part of a general turnover process termed 'haematopoiesis') and this process requires the cells to be directed to various sites within the body as part of the differentiation/maturation process (incidentally, T cells are produced in the thymus and B cells in the bone marrow).

Therefore, this localisation of leukocytes (and other cell types) is of fundamental importance in the immune system, and a lack of cell mobility will result in an ineffectual immune system. There are a number of molecules that have been demonstrated to play a role in this localisation process, among them selectins, integrins, and the non-specific chemoattractants C5a, fMLP and prostaglandins (PGs). However, the most significant group of molecules implicated in this directed cell migration are those of the chemokine family, and this group will be the focus of this thesis.

1.2 Chemokines & Their Receptors

History

Up until the late 1980s (1987-'88), the existence of chemokines was unknown and the phenomenon of specific leukocyte subtype migration during various disease processes was hard to explain. Prior to 1987, only molecules such as C5a and formyl peptide were known for their attraction of leukocytes, unspecifically. This all changed in 1987, firstly with the discovery of the cytokine Interleukin-8 (IL-8) (Walz et al., 1987; Yoshimura et al., 1987a; Yoshimura et al., 1987b) and, followed closely on its heels, with Macrophage Inflammatory Protein-1 α (MIP-1 α) (Wolpe et al., 1988). Within a few years a large number of these molecules were discovered. As a consequence, the term chemokine was coined to describe these chemotactic cytokines at the 3rd International Symposium on Chemotactic Cytokines at Baden, Germany in 1992 (Lindley et al., 1992), where two groups of chemokines were identified – alpha and beta (CXC and CC, respectively).

Today, a PubMed search for the word “chemokine” would result in more than 22000 hits, with a vast number coming in the last 5 years (table 1.1), reflecting the discovery of an ever-increasing role of chemokines in diseases. However, the last 2-3 years has seen the number of papers produced plateau, suggesting that the chemokine field has now effectively reached maturity.

Table 1.1. Number of papers published each year since 1991.

Year	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003
No. of Papers	291	361	511	621	800	1076	1456	1721	2018	2239	2745	2798	2626

Papers published with the word “chemokine” in the title or abstract. PubMed used as search engine.

Nomenclature and Structural Characteristics of Chemokines

Chemokines share a limited sequence homology but possess similar secondary and tertiary structures. This similar structure is known as the ‘Greek-key’ motif – three anti-parallel beta-pleated sheets overlaid by a C-terminal alpha helix (Chung et al., 1995; Clore et al., 1989). Chemokines are in the range of 8-11 kDa in size and bind to one of four classes of

chemokine receptor, normally achieving biological functions at nanomolar concentrations.

These four classes are:

- CC Receptors (CCR)
- CXC Receptors (CXCR)
- XC Receptor (XCR)
- CX3C Receptor (CX3CR)

Silent Receptors

In addition to these 4 classes of chemokine receptors, there are two chemokine-binding proteins called Duffy (DARC (Duffy Antigen Receptor for Chemokines)) (Horuk et al., 1993) and D6 (Nibbs et al., 1997) that bind various chemokines but appear not to signal and, as a consequence, will not be discussed in any detail (for a review see (Pogo and Chaudhuri, 2000)). However, their role may be to act as a sink for inflammatory chemokines, thereby modulating the inflammatory response (Fra et al., 2003; Kashiwazaki et al., 2003).

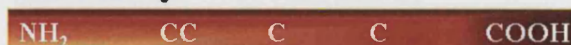
Though the chemokines are specific for a particular subclass of receptor there are several chemokines that possess the ability to bind to more than one receptor subtype, for example, Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES) is able to bind CCR1, CCR3 and CCR5 (table 1.2). However, the chemokines Macrophage-Derived Chemokine (MDC) and Thymus- and Activation-Regulated Chemokine (TARC) only bind CCR4. To date there are over 40 recognised chemokines and many of these have more than one name, e.g. Pulmonary and Activation-Regulated Chemokine (PARC) had at least three other names (AMAC-1, MIP-4 and DC-CK1) (Murphy et al., 2000). These names arose due to various research groups independently identifying chemokines and naming them according to the protein's function. Recently, to overcome the confusion that can arise with different groups calling the same chemokine different names, the international chemokine community has developed a new approved nomenclature where the chemokines are named according to the arrangement of their two highly conserved

Table 1.2. Systematic chemokine nomenclature and characteristics.

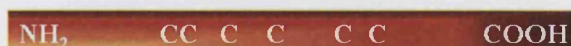
Systematic Name	Common Synonyms	Human Chromosome	Chemokine Receptors Bound	Chemokine Type	Expression
CC Chemokine/Receptor (β) Family					
CCL1	I309	17q11.2	CCR8	4 cysteines	Inducible
CCL2	MCP-1	17q11.2	CCR2	4 cysteines	Inducible
CCL3	MIP-1 α	17q11.2	CCR1/CCR5	4 cysteines	Inducible
CCL4	MIP-1 β	17q11.2	CCR5	4 cysteines	Inducible
CCL5	RANTES	17q11.2	CCR1/CCR3/CCR5	4 cysteines	Inducible
CCL6	?	?	?	4 cysteines	?
CCL7	MCP-3	17q11.2	CCR1/CCR2/CCR3	6 cysteines	Inducible
CCL8	MCP-2	17q11.2	CCR2/CCR3	4 cysteines	Inducible
CCL9	?	?	?	6 cysteines	?
CCL10	?	?	?	4 cysteines	?
CCL11	Eotaxin	17q11.2	CCR3	4 cysteines	Inducible
CCL12	?	?	?	4 cysteines	?
CCL13	MCP-4	17q11.2	CCR2/CCR3	4 cysteines	Inducible
CCL14	HCC-1	17q11.2	CCR1	4 cysteines	Constitutive
CCL15	HCC-2/Lkn-1	17q11.2	CCR1/CCR3	6 cysteines	Constitutive
CCL16	HCC-4/LEC	17q11.2	CCR1	4 cysteines	Constitutive
CCL17	TARC	16q13	CCR4	4 cysteines	Both
CCL18	PARC/DC-CK1/AMAC	17q11.2	Unknown	4 cysteines	Constitutive
CCL19	MIP-3 β /ELC	9p13	CCR7/CCR11	4 cysteines	Constitutive
CCL20	MIP-3 α /LARC	2q33 – q37	CCR6	4 cysteines	Both
CCL21	6CKine/SLC	9p13	CCR7/CCR11	6 cysteines	Constitutive
CCL22	MDC/STCP-1	16q13	CCR4	4 cysteines	Both
CCL23	MPIF-1/MIP-3	17q11.2	CCR1	6 cysteines	Inducible
CCL24	MPIF-2/Eotaxin-2	7q11.23	CCR3	4 cysteines	Inducible
CCL25	TECK	19p13.2	CCR9/CCR11	4 cysteines	Constitutive
CCL26	Eotaxin-3	7q11.23	CCR3	4 cysteines	Inducible
CCL27	CTACK/ILC/ESkine	9p13	CCR10	4 cysteines	Constitutive
CCL28	MEC	5p12	CCR3/CCR10	6 cysteines	Constitutive
CXC Chemokine/Receptor (α) Family					
CXCL1	Gro α /MGS α - α	4q12 – q13	CXCR2	ELR +	Inducible
CXCL2	Gro β /MGS α - β	4q12 – q13	CXCR2	ELR +	Inducible
CXCL3	Gro γ /MGS α - γ	4q12 – q13	CXCR2	ELR +	Inducible
CXCL4	PF-4	4q12 – q13	Unknown	ELR –	?
CXCL5	ENA-78	4q12 – q13	CXCR2	ELR +	Inducible
CXCL6	GCP-2	4q12 – q13	CXCR1/CXCR2	ELR +	Inducible
CXCL7	NAP-2	4q12 – q13	CXCR2	ELR +	Inducible
CXCL8	IL-8	4q12 – q13	CXCR1/CXCR2	ELR +	Inducible
CXCL9	Mig	4q21.21	CXCR3	ELR –	Inducible
CXCL10	IP-10	4q21.21	CXCR3	ELR –	Inducible
CXCL11	ITAC	4q21.21	CXCR3	ELR –	Inducible
CXCL12	SDF-1 α/β	10q11.1	CXCR4	ELR –	Constitutive
CXCL13	BLC/BCA-1	4q21	CXCR5	ELR –	Constitutive
CXCL14	BRAK/Bolekine	5q31	Unknown	ELR –	Inducible? ¹
CXCL15	Lungkine	Unknown	Unknown	ELR –	?
CXCL16	SR-PSOX	17p13	CXCR6	ELR –, TMD +	Inducible?
C Chemokine/Receptor (γ) Family					
XCL1	Lymphotactin α /ATAC	1q23	XCR1	2 cysteines	Both
XCL2	Lymphotactin β	1q23	XCR1	2 cysteines	Both
CX₃C Chemokine/Receptor (δ) Family					
CX3CL1	Fractalkine/neurotactin	16q13	CX3CR1	TMD +	Both

Legend for Table 1.2. *Abbreviations:* ELR +/-, presence or absence (respectively) of the tripeptide motif glutamic acid-leucine-arginine in the NH₂ – terminal region of CXC chemokines; TMD, transmembrane domain; ¹, only a single paper published (Mitsui et al., 2003). See “abbreviations” for chemokine acronyms.

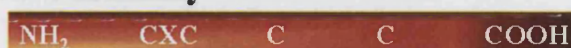
amino-terminal cysteine residues. Four cysteines are present in total, except Lymphotactin that has only the first and third cysteines of the four-cysteine motif (a few members of the CC family possess an extra 2 cysteines (6 in total) that can further be used to subdivide the CC family (fig. 1.2, table 1.2)). Disulphide bonds form between the first and third cysteines and between the second and fourth, giving the chemokines their characteristic three-dimensional folding mentioned earlier. In the CXC family a single amino acid is interposed between the two amino-terminal cysteines, whilst in the CC family the cysteines are adjacent. As a result, PARC becomes CCL18, MDC becomes CCL22 and RANTES is now CCL5 (table 1.2). However, this thesis will use the acronyms MDC and TARC for the CCR4 ligands (instead of CCL22 and CCL17, respectively), due to their greater familiarity. There are currently 16 chemokines in the CXCL group, 28 in the CCL class and just two in the XCL (Lymphotactin 1 & 2) and one in the CX3CL (Fractalkine (three amino acids separate the two cysteines)) groups. Fractalkine is interesting in that it is tethered to the cells that produce it via a mucin-like stalk (Bazan et al., 1997), thereby promoting leukocyte adhesion (the newly identified CXCL16 also has a membrane-anchored form (Matloubian et al., 2000)). The CXCL group can further be sub-categorised into two groups depending on the presence of the ELR (Glu-Leu-Arg) motif preceding the first cysteine. CXCR1 and CXCR2 binding chemokines (e.g. IL-8) are ELR-positive, while the other CXCR-binding chemokines (e.g. IP-10) lack this motif. (Strieter et al., 1995a). ELR + chemokines tend to, therefore, be specific for myeloid cells (mainly neutrophils), whilst ELR– attract a variety of leukocytes. These four chemokine families tend to be clustered at four separate human chromosomal loci: 4q12-21 for CXC (exceptions include SDF-1/CXCL12 at 10q11 and CXCL14 at 5q31); 17q11.2-21 for CC (notable exceptions are TARC/CCL17 and MDC/CCL22 both at 16q13); 1q23 for XCL; and 16q13 for CX3CL1 (Murphy et al., 2000) Chemokines that bind to the same receptors tend to have the same chromosomal location, e.g. CXCR3 ligands are all at 4q21.21.

CC Subfamily

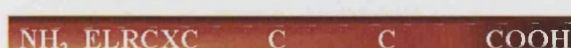
4 cysteine members



6 cysteine members*

CXC Subfamily

ELR- members



ELR+ members



CXCL16

CC Subfamily**CX3C Subfamily**

Figure 1.2. General structure of chemokine superfamily members. Schematic representation of the cysteine signature motifs that divide the family into 4 subfamilies. Further subdivisions can be made within the CXC and CC families according to the presence or absence of an ELR motif immediately preceding the CXC motif, or the presence of either four or six cysteines in the CC members. * most common arrangement of extra cysteines depicted (in some cases the second extra cysteine can appear after the fourth conserved cysteine). *Abbreviations:* TM, transmembrane; M-L S, mucin-like stalk.

Chemokines are synthesised with a prosequence of approximately 20 amino acids that is cleaved off before secretion (except for Fractalkine), and all possess a positive charge meaning that they bind to proteoglycans on cell surfaces or the extracellular matrix (Kuschert et al., 1999; Tanaka et al., 1993; Webb et al., 1993). This ensures that the chemokines remain concentrated locally, forming stable gradients and, in some cases, enhances potency (Schall and Bacon, 1994). In addition, chemokines are able to form dimers in concentrated solutions (much higher than required for biological activity and will dissociate when diluted), with the exception of a few, e.g. MCP-3 which is always monomeric. Monomeric forms possess full biological activity (Baggiolini et al., 1997; Horcher et al., 1998; Kim et al., 1996; Lowman et al., 1997). It has been suggested that, in

vivo, chemokines can form tetrameric structures and that these may be promoted by the binding to glycosaminoglycans (Swaminathan et al., 2003).

Chemokine Receptor Structure

All chemokines bind to a branch of the rhodopsin family of seven transmembrane G-protein coupled receptors (Class A (6 GPCR classes in total (Qian et al., 2003))) – which have a size of about 40 kDa (340 – 370 amino acids), and classified according to the type of chemokine they bind (table 1.3). The heptahelical chemokine receptors also share the highly conserved sequence motifs characteristic of the rhodopsin-like family of GPCRs, with the majority of these located in the transmembrane regions. This suggests the conservation of a common fold throughout the rhodopsin family (Strosberg, 1991). However, there is broad structural diversity among the extracellular regions. These features result in a 25-80% homology among all known chemokine receptors (fig. 1.3). Chemokine receptors possess an extracellular N-terminus, seven hydrophobic transmembrane regions, three extracellular and intracellular loops, and a cytoplasmic C-terminus “tail”.

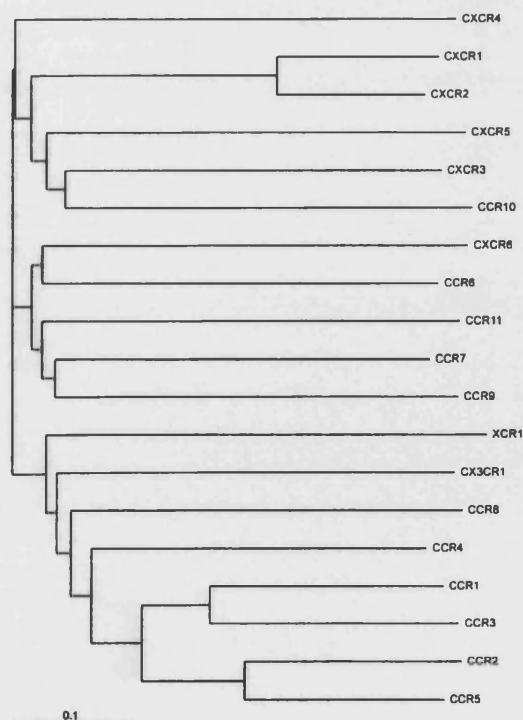


Figure 1.3. Dendrogram showing the amount of protein sequence similarity among all known human chemokine receptors. The scale bar reflects the horizontal distance at which sequences diverge by 10% (90% identity). Amino acid identity (%) between a pair of chemokine receptors is given by $1 - x$, where x is the sum of the 2 horizontal distances to the right of the pair's vertical branch point. For example, the horizontal distances before the vertical branch point of CXCR1 and CXCR2 are 12.9 and 11.8%, respectively. Therefore, the amino acid identity between these chemokine receptors is $100 - (12.9 + 11.8)\%$ or 75.3%. (Reproduced from (Olson and Ley, 2002)).

Table 1.3. Characteristics of chemokine receptors.

Chemokine Receptor	Chemokine Ligands	Cellular Distribution	Presumed Function	Therapeutic Potential
CC				
CCR1	CCL3, 5, 7, 14, 15, 16, 23	NK, Th1, Th2, iDC, M, MΦ, Ba, Eo, N	Th1 response	MS, RA, GVHD
CCR2	CCL2, 7, 8, 13, 16	NK, Th1, Th2, M, Ba, iDC	Inflammation	MS, RA, A, GN, AS
CCR3	CCL5, 7, 8, 11, 13, 15, 24, 26, 28	Th2, Ba, Eo, P, DC	Th2 response	A, CD
CCR4	CCL17 (TARC), CCL22 (MDC)	Thy, NK, Th2, Tc2, Tm, Ba, P, iDC	Th2 response	S, A, CD, AD
CCR5	CCL3, 4, 5, 8, 14	M, MΦ, Thy, Th1, Tc1, iDC	Th1 response	MS, RA, II, GVHD, HIV
CCR6	CCL20	Tm, B, iDC, M, MΦ.	DC function	Ps
CCR7	CCL19, 21	Thy, Tn, B, mDC.	DC/ LΦ migration to lymph nodes	C
CCR8	CCL1	Thy, Th2, M, N, B	Th2 response	AD, A
CCR9	CCL25	Thy, Tm, B	LΦ trafficking in thymus & SI	II
CCR10	CCL27, 28	Tm	LΦ trafficking in skin & colon	UC, SKI
CCR11	CCL19, 21, 25	T, iDC	Not known	Not identified
CXC				
CXCR1	CXCL6, 8	N, T, M, MΦ, DC	N. recruitment	LRI, Gout, Ps, C
CXCR2	CXCL1, 2, 3, 5, 6, 7, 8	N, T, M, MΦ, DC, Eo	N. recruitment & angiogenesis	LRI, Gout, Ps, C, AS
CXCR3	CXCL9, 10, 11	N, Th1, B, DC, Eo, P	Th1 response & angiostasis	MS, RA, GVHD, C, SD
CXCR4	CXCL12	N, M, MΦ, T, B, DC, P	Organogenesis	HIV, C
CXCR5	CXCL13	M, MΦ, T, B	B cell migration	Not identified
CXCR6	CXCL16	T, DC	Not known	Not identified
C				
XCR1	CX3CL1	T	Not known	Not identified
CX3CR1	XCL1, 2	N, M, MΦ	Cell adhesion to endothelia/neurons	GVHD, GN, CNSi

Ligands CCL6, CCL9/10, CCL18, CXCL4, CXCL14, CXCL15 were omitted, as the corresponding receptor(s) have not been identified. *Colour coding:* blue = inflammatory/inducible chemokines; yellow = homeostatic/constitutive chemokines; green = both inflammatory and constitutive chemokines. *Abbreviations for cell types:* B, B cell; Ba, basophil; DC, dendritic cell; iDC, immature dendritic cell; mDC, mature dendritic cell; Eo, eosinophil; LΦ, lymphocyte; M, monocyte; MΦ, macrophage; N, neutrophil; NK, natural killer cell; P, platelet; SI, small intestine; T, T-cell; Th, T-helper cell; Thy, thymocytes; Tc, cytotoxic T cells; Tm, memory T cells; Tn, naïve T cells. *Abbreviations for diseases:* A, asthma; AD, atopic dermatitis; AS, atherosclerosis; C, cancer; CD, contact dermatitis; CNSi, central nervous system inflammation; GN, glomerulonephritis; GVHD, graft-versus-host disease; II, intestinal inflammation; LRI, lung reperfusion injury; MS, multiple sclerosis; Ps, psoriasis; RA, rheumatoid arthritis; S, sepsis; SD, sarcoidosis; SKI, skin inflammation; UC, ulcerative colitis.

The chemokine receptors share a high structural homology in certain areas, most notably with conserved sequences in the second and seventh transmembrane domains:

TM2 = ADLLFxxTLPFW

TM7 = HCCxNPxxY

In addition to these transmembrane domains, there is also a conserved sequence, DRYLAIVH, in the second intracellular loop (loop 3) domain that is adjacent to the third transmembrane region and is involved in G protein coupling (Power and Wells, 1996; Rollins, 1997); a significant number of acidic amino acid residues within the 30+ long amino acid extracellular N-terminus; and two extracellular disulphide bonds created from the 4 single cysteine residues in each of the extracellular domains (N-terminal – second loop and first loop – third loop). The extracellular domain consists of the N-terminus and three extracellular loops (loops 2, 4 and 6), whilst the remaining loops (loops 1, 3, 5) and the C-terminus are intracellular. Structure-activity studies with CXC and CC chemokines have revealed that they have two major sites of interaction with their receptors: an amino-terminal domain that acts as a *triggering domain* (also known as the ‘message’ in the ‘two-site hypothesis’ model), activating the receptor; and a domain in the exposed loop after the second cysteine that acts as a *docking domain* (or ‘address’), restricting chemokine mobility allowing for the correct orientation of the triggering domain and responsible for receptor specificity/affinity. The two-disulphide bonds keep both domains in close proximity (fig. 1.4) (Baggiolini, 2001).

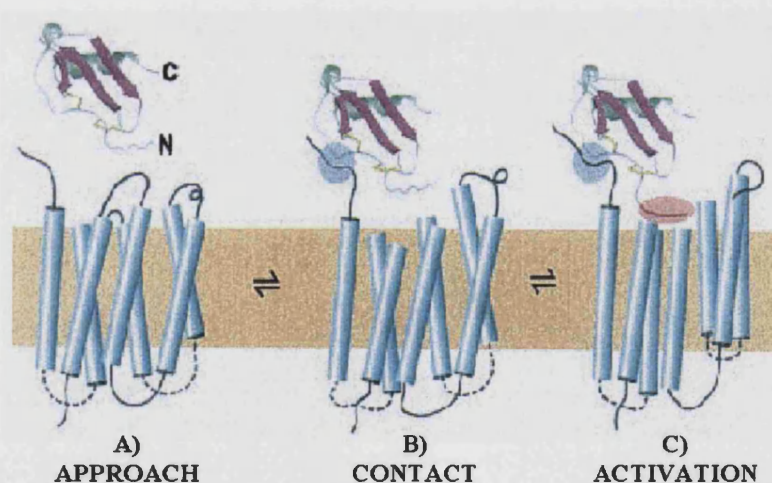


Figure 1.4. Model of the interaction between a chemokine and its receptor. The scheme indicates that chemokines interact first with their docking and then with their triggering site, example given is for SDF-1/CXCL12 docking with CXCR4 (reproduced from (Baggiolini, 2001) initially published by (Crump et al., 1997)). B) indicates interaction of CXCL12 with the N-terminal segment of the receptor (blue), followed by C), binding of the N-terminal region of CXCL12 with the groove (pink) above the helices leading to activation of the receptor (depicted as a conformational change).

A number of chemokine receptors demonstrate remarkable promiscuity for the chemokines they bind. Although the receptors will only bind chemokines of their own class (i.e. CXCRs only bind CXCs), receptors such as CCR3 can bind up to 9 different chemokines (table 1.3). This is discussed in the 'Receptor Promiscuity' section in more detail.

All known chemokine receptors are coupled to the *Bordetella pertussis* toxin sensitive G α i proteins (excluding the 'Silent Receptors'). Activated G α i subunits of heterotrimeric G proteins inhibit adenylate cyclase. G $\beta\gamma$ subunits make up the heterotrimeric complex (See the "Signal Transduction Pathways" section for more detail). Currently, there are 11 CCRs, 6 CXCRs and a solitary CX3CR and XCR. Generally, very little research has been carried out on these individual receptors. The two exceptions to this are CCR5 and CXCR4 that were found to be co-receptors for HIV in the middle of the 1990s (Bleul et al., 1996; Deng et al., 1996; Feng et al., 1996; Oberlin et al., 1996). As would be expected, this led to a substantial increase in the study of these two receptors (table 1.4).

Table 1.4. Number of papers published for each chemokine receptor subtype.

Receptor Subtype Receptor Class	1	2	3	4	5	6	7	8	9	10	11
XCR	10	-	-	-	-	-	-	-	-	-	-
CXCR	192	316	362	1922	82	49	-	-	-	-	-
CX3CR	108	-	-	-	-	-	-	-	-	-	-
CCR	540	487	537	330*	2523	185	324	129	42	28	6

Number of papers published for each individual chemokine receptor that is mentioned in the title or abstract, up until 31st December 2003. PubMed used as search engine.

* Includes papers associated with CCR4-NOT complex research (estimated total of CCR4 chemokine papers is 247 (search = CCR4 + chemokine).

1.3 Chemokine & Receptor Biology

Features

Inflammatory vs. Constitutive Chemokines

Every year, chemokines are found to play a role in an ever increasing number of pathological conditions and, depending on their function in immunity and inflammation,

can be classified into two groups: *inflammatory chemokines* and *homeostatic chemokines* (figure 1.5). Inflammatory (inducible) chemokines are produced by many different cells (by both resident (e.g. endothelial and epithelial cells) and immigrating cells (e.g. leukocytes)) in inflamed tissue, in response to pro-inflammatory cytokines and bacterial toxins, with mRNA levels able to increase over 300-fold within a few hours of activation (Gerard and Rollins, 2001). These chemokines function to recruit effector cells, such as monocytes, granulocytes, and effector T cells, for host defence in infection and immunity. Conversely, homeostatic (constitutive/housekeeping/lymphoid) chemokines are expressed constitutively in defined microenvironments within lymphoid tissue and in the skin and mucosa. These chemokines are responsible for maintaining homeostatic leukocyte traffic and cell compartmentalisation within secondary lymphoid tissues. As a consequence, they are involved in relocation and recirculation of lymphocytes in maturation, differentiation and activation, therefore playing a role in the innate immune system (Baggiolini, 2001; Moser and Loetscher, 2001; Youn et al., 2000). In general, homing chemokine receptors are selective for no more than two ligands and inflammatory chemokine receptors are able to bind multiple ligands, with the exception of CXCR1 (IL-8 & GCP-2).

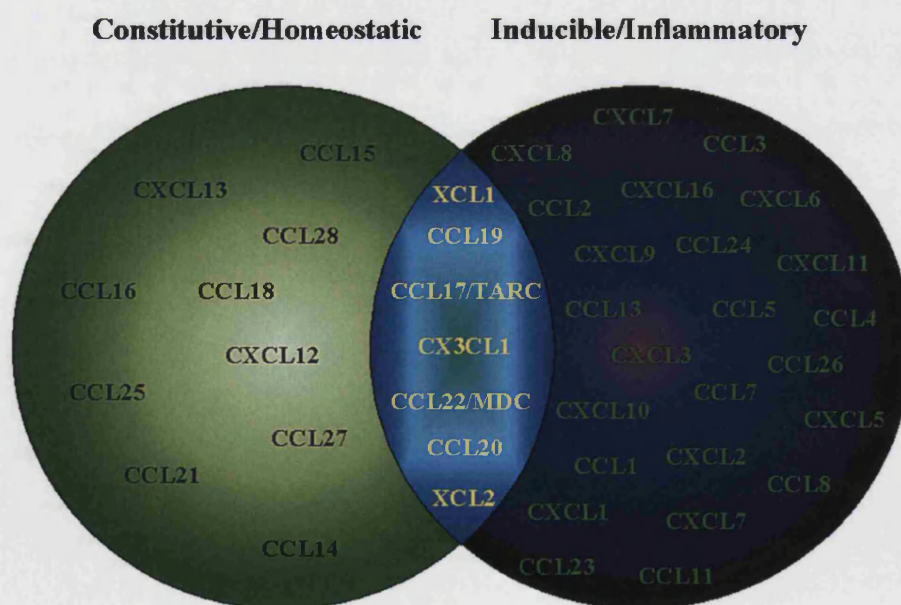


Figure 1.5. The functional classification of chemokines into inflammatory, homeostatic and those that belong to both subfamilies.

However, this division of the receptors/ligands should not be regarded as absolute. It is clear, subsequent to reading the many chemokine reviews, that this classification varies from paper-to-paper, especially with regard to a few chemokines and where they should be placed (these include CX3CL1, CCL19, CCL28, CCL17, XCL1, XCL2). It is perhaps more accurate to use the terms constitutive and inducible rather than homeostatic or inflammatory, respectively. For example, CCR7/CCL19/CCL21 participate in the presentation of antigen to T cells due to its expression on B cells and dendritic cells (DCs), and even then CCL19 production is induced in maturing DCs and CCL18 production is massively upregulated (both generally considered to be constitutive, although CCL18 is constitutively transcribed in DCs before an increase in the levels of induction) (Sallusto et al., 1999b).

The ELR Motif – A Further Sub-Classification of CXC Chemokines

The CXC subfamily of chemokines can further be divided into two groups depending upon the presence of the tripeptide motif glutamic acid-leucine-arginine (ELR) preceding the first amino-terminal cysteine (table 1.2; fig. 1.2). Although ELR+ chemokines (ELR-CXC) tend to be specific for myeloid cells and the ELR– chemokines attract a variety of leukocytes; it is more interesting to note the opposing effects the two groups have on angiogenesis. Angiogenesis is the biological process through which blood vessels are generated. The ELR-CXC chemokines have been demonstrated to be very potent angiogenic factors, whilst the ELR-deficient CXC chemokines are strong angiostatic factors that inhibit the endothelial cell migration induced by ELR-CXC chemokines (Koch et al., 2001; Strieter et al., 1995b). This discovery has led to the observation that the balance between angiogenic and angiostatic chemokines appears to be altered in several diseases that will be discussed in the “Chemokines & Pathology” section.

Chemokine Receptors as Markers of Leukocytes and Subsets

Chemokine receptors are expressed on a large number of different cells in a wide variety of tissues (table 1.3), as are the chemokines produced by a variety of cells. However, certain leukocytes express certain chemokine receptors and subsets of different leukocytes display varying receptor profiles. This allows cells to be characterised according to their chemokine receptor expression profile (fig. 1.6). For instance, T helper cell subsets can be distinguished by the presence or absence of CXCR3, CCR4, CCR8; immature DCs can be defined from mature DCs by the presence of CCR1, CCR5, and CCR6 (with mature DCs additionally inducing the expression of CCR7). This allows for an efficient immune system where type of cell and its maturity defines its localisation and role within the immune response (this is reviewed in depth in the imminent “Chemokines and Their Physiological Role” section).

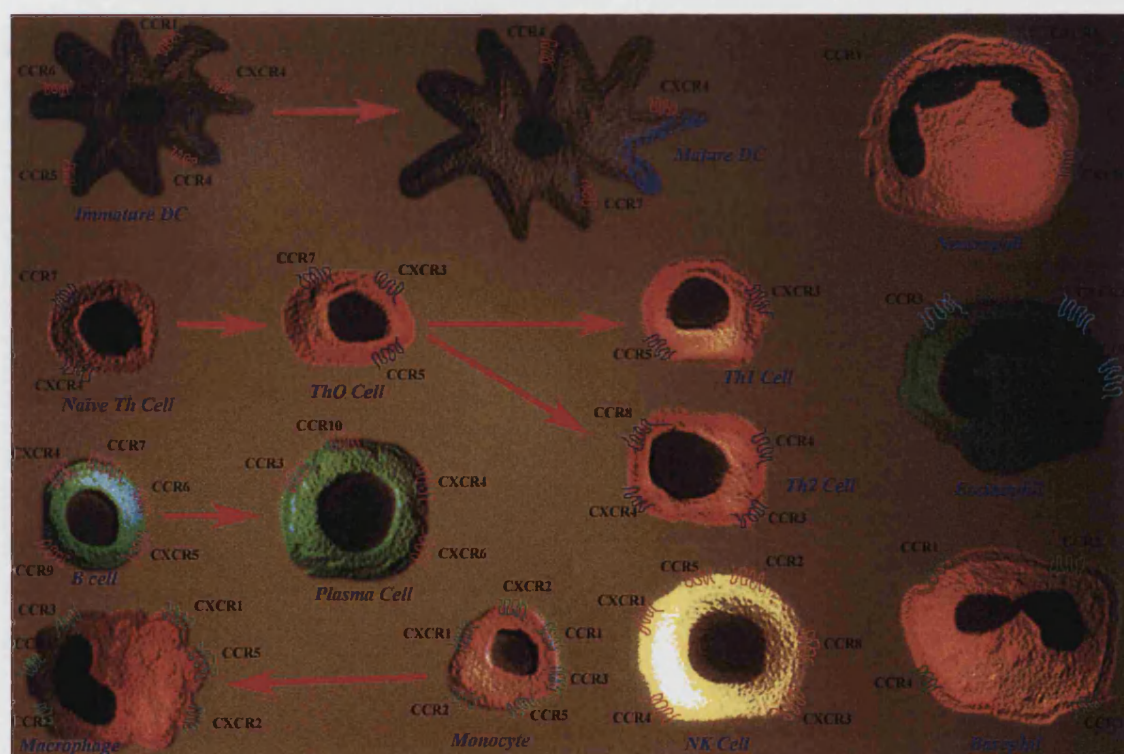


Figure 1.6. Overview of chemokine receptor expression on leukocytes. Represents a simplified view of some of the more important chemokine receptors on each cell type and the differences observed within subsets (e.g. the T cell subset). *Abbreviations:* NK, natural killer T cell; DC, dendritic cell; Th, helper T cell.

Receptor Promiscuity

As mentioned earlier, most chemokine receptors can bind more than one chemokine in its class, e.g. CCR3 binds with high affinity no less than 9 CCL chemokines. Receptor promiscuity correlates generally with whether the chemokine is considered to be inflammatory or homeostatic, with the homeostatic (constitutive) chemokines generally having a “monogamous” relationship with their receptor (to be more precise, one or two ligands per receptor) and the receptors such as CCR1-3, 5 and CXCR1-2 binding multiple inflammatory chemokines (table 1.3). This is likely to be a result of evolutionary pressures, with the proliferation of inflammatory chemokines due to the ongoing battle with pathogens and the need to generate a more robust chemokine response (a result of redundancy in the system and is highlighted by knockout studies that are mentioned later). This is supported by the fact that species-specific chemokines are found normally within the inflammatory chemokines, yet the homeostatic chemokines exhibit a very conserved phenotype between mouse and human. Additionally, the genes encoding inflammatory chemokines tend to be present in clusters (CC = chr. 17; CXC = chr. 4), with homeostatic chemokine genes found in isolated chromosomal locations. This indicates that the homeostatic chemokines are under pressure not to diverge due to a critical function for the organism. The inflammatory chemokines cluster their genes suggesting that these are newer developments in evolution and are likely to share similar functions (A. Zlotnik, lecture, Chemokines II conference, Paris, Oct. 23, 2003). Robustness in the chemokine system is discussed in more depth in a review of chemokines by Alberto Mantovani (Mantovani, 1999).

Chemokines & Their Physiological Role

Chemokines and their receptors are involved in a variety of physiological processes (fig. 1.7). The ELR-CXC chemokines are involved in the formation of new blood vessels that, along with other processes, are essential for effective wound healing (Gillitzer and Goebeler, 2001). During maturation, B and T cells regulate several chemokine receptors allowing these cells to traffic from bone marrow to spleen and then to other lymphoid tissue

microenvironments (Annunziato et al., 2001; Bleul et al., 1998; Bowman et al., 2000; Hernandez-Lopez et al., 2002; Uehara et al., 2002). This discriminating migration is helped by the selective expression of chemokines within the lymphoid organs (Rossi and Zlotnik, 2000). Chemokines are also integral for effective immunity by both the innate and adaptive systems, involved in immune surveillance and leukocyte differentiation (Campbell et al., 2003; Kunkel et al., 2003; Luster, 2002; Luther and Cyster, 2001). Some receptors are essential for life, e.g. CXCR4 is required for embryogenesis and organogenesis. This was demonstrated with CXCR4 knockout models displaying defects in cardiogenesis and subsequent foetal lethality (chemokine knockout models are discussed in the following “Chemokines & Pathology” section) (Ma et al., 1998; Tachibana et al., 1998; Zou et al., 1998). Conversely, the requirement for some chemokine receptors, most notably CCR5, appear to be redundant as highlighted by the fact that this chemokine receptor is functionally missing in some individuals without any apparent consequences in the individuals health, but its loss can provide a degree of protection from HIV (Dean et al., 1996; Huang et al., 1996; Liu et al., 1996; Martinson et al., 1997; Samson et al., 1996a). There are a number of papers reviewing the biological functions of chemokines in much more detail than reviewed here (Mackay, 2001; Rossi and Zlotnik, 2000).

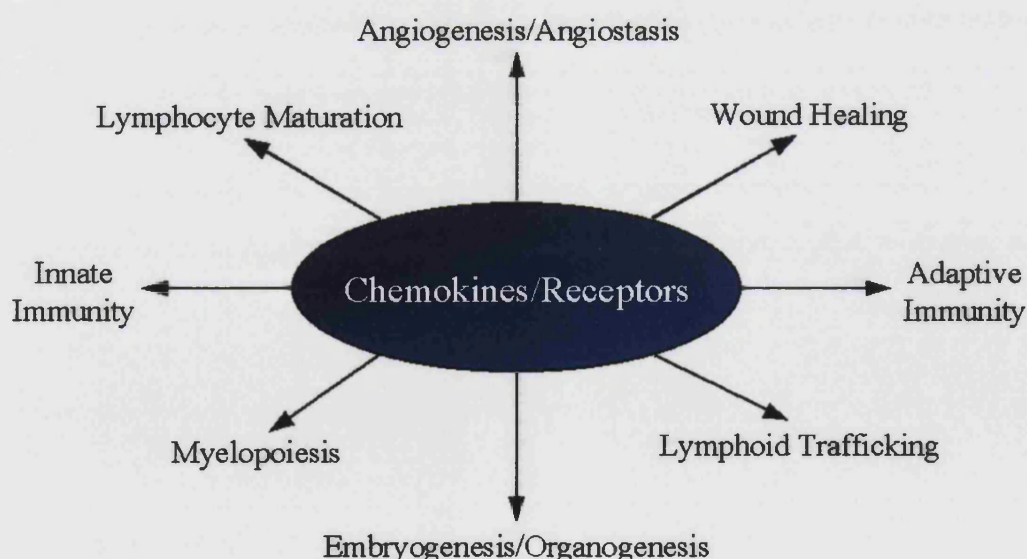


Figure 1.7. Physiological functions of chemokines and chemokine receptors.

Naïve T cells (and other leukocytes) continuously recirculate from the blood to lymph nodes and other secondary lymphoid tissues during immune surveillance, returning to the blood via the lymphatics. Activated T cells, and other leukocytes, are additionally recruited from the blood to sites of inflammation and tissue damage. A key process in this pathway is the extravasation of the leukocyte in to the surrounding tissue from the blood vessels, with chemokines playing a pivotal role.

Leukocyte Extravasation

Intravascular leukocytes are exposed to extraordinarily high shear stresses within the mainstream of blood and, without adequate equipment, would be extremely difficult to exit the flow, much like an individual attempting to exit a raging river after falling in. Fortunately, leukocytes have a well-coordinated and efficient mechanism of extravasation from the blood vessel. Von Andrian and Mackay (Von Andrian and Mackay, 2000) made an enlightening comment about the extent of the physical conditions the leukocytes are under in the blood stream: “the jet d’eau fountain in Lake Geneva spouts 500 litres of water per second with a mean velocity of 200 km per hour, reaching a height of 140 m. Assuming that a cross-section of the water column is circular, the wall shear stress at the nozzle equals approximately 41.5 dyn per square centimetre.” Compare this to a shear stress of up to 50 dyn per square centimetre for the flow of blood in a vessel.

In order to enable a better efficiency of extravasation, leukocytes usually attach to post-capillary venules where the shear stress is the lowest (Alon et al., 1998). The initial step in leukocyte extravasation is the tethering/rolling of marginated leukocytes to the endothelium via transient interactions with selectins (at highest density on microvillous surface protrusions – L- and P-selectins being most effective) that bind ligands modified with specific carbohydrate epitopes (fig.1.8) (Vestweber and Blanks, 1999; Von Andrian et al., 1995). L-selectin is expressed constitutively on leukocytes and recognises sulphated sialyl-Lewis (sLe^x)-like sugars (called peripheral-node addressin (PNAd)) in high endothelial venules; E- and P-selectin are upregulated by activated endothelium in response to inflammatory stimuli and bind P-selectin glycoprotein ligand 1 (PSGL-1) and free sLe^x-like glycans. PSGL-1 binding to P-selectin requires sLe^x-like sugars to be close to a tri-

tyrosine sulphated motif in the N-terminal. The $\alpha 4$ integrins (activation-independent) are also able to support leukocyte rolling and tethering (Alon et al., 1995; Bargatze et al., 1995; Berg et al., 1993; Berlin et al., 1995).



Figure 1.8. The process of leukocyte extravasation. The movement of leukocytes from the microvasculature to the area of pathogen in the surrounding tissue involves: tethering; rolling; activation of integrins; arrest (firm adhesion); diapedesis; and chemotaxis. *Abbreviations:* sLe^x, sialylated Lewis X – like glycans; Y, tyrosine sulphated motif; LPS, lipopolysaccharide; PSGL-1, P-selectin glycoprotein ligand 1; PNAd, peripheral-node addressin; MAdCAM-1, mucosal addressin-cell adhesion molecule 1; VCAM-1, vascular-cell adhesion molecule 1; ICAM, intercellular adhesion molecule.

In order for firm adhesion to occur, the integrins (in their low-affinity state for ligands) must become activated. Specific GPCRs on the leukocytes respond to chemoattractants, such as chemokines, PAF, LTB₄, C5a and formyl peptides, displayed by endothelial cells where they cause the rapid activation of β2 and/or α4 integrins which then bind to members of the endothelial immunoglobulin superfamily. Although most chemokines are secreted (except CX3CL1 and CXCL16) it is unlikely that they would persist at the blood-endothelium interface due to the shear flow. Therefore, it is likely that the chemokines

bind to GAGs leading to the immobilisation of chemokines on the endothelial cell surface (Amara et al., 1999; Tanaka et al., 1993; Webb et al., 1993).

Once the leukocyte has arrested it then transmigrates across the endothelium and in to the tissue, a process termed 'diapedesis'. Diapedesis is a technique in which the leukocyte, via rapid disassembly of its cytoskeleton, crawls between tightly apposed endothelial cells by forming a pseudopod, and reassembles itself on the abluminal side of the endothelium. Unlike the processes mentioned so far, where all the interactions have been heterophilic, diapedesis relies on at least 2 major homophilic interactions involving platelet-endothelial-cell adhesion molecule-1 (PECAM-1/CD31) and CD99 (Schenkel et al., 2002). These 2 molecules and along with another 4 (VE-cadherin, JAM-A, -B, -C) are thought to play major roles in diapedesis (JAM-A and -C are also expressed on leukocytes and may take part in homophilic interactions) (Muller, 2003). Blocking homophilic PECAM interaction blocks diapedesis with no pseudopods entering the intercellular junction (Liao et al., 1995; Liao et al., 1997). Diapedesis also requires a transient increase in intracellular free calcium within the endothelial cells the leukocyte is adjacent to (Huang et al., 1993). The increase in intracellular calcium activates myosin light chain kinase leading to a conformational change in myosin II and subsequent contraction of actin-myosin bundles. This leads to the retraction of the endothelial cells facilitating leukocyte passage (Hixenbaugh et al., 1997; Saito et al., 1998). Once the cell has squeezed through the endothelium it then becomes susceptible to chemokine gradients, leading to chemotaxis of the cell to specific locations in the tissue.

Chemotaxis

Chemotaxis is the directional cell movement up a chemoattractant gradient. In order for a cell to chemotax it requires dynamic and spatially regulated changes to the cytoskeleton and cell adhesion. This results in a defined cell polarity in which the cytoskeletal components are differentially localised at two poles of the cell, determining the front and rear. Cell migration is a multi-step process that can be divided into four mechanistically distinct steps (Lauffenburger and Horwitz, 1996):

- 1) Lamellipodium extension,
- 2) New adhesion formation,
- 3) Cell body contraction,
- 4) Tail (uropod) detachment & retraction.

Lamellipodium extension requires actin polymerisation, with lamellipodia consisting of branching networks of F-actin. Lamellipodium can vary in breadth from 1 – 5 μm depending on cell type and can exhibit variable numbers of radiating bundles 0.1 – 0.2 μm in diameter and many μm long (microspikes or filopodia) (Small et al., 2002). This lamellipodia extension is then stabilised through the formation of new adhesions to the extracellular matrix. These small focal complex structures (involving integrins) are localised to the lamellipodia and it is important that focal complexes/adhesions turn over for cells to migrate. This is demonstrated with the inhibition of cell migration when there is a high-level of integrin-mediated adhesion (due to strength of attachment) (Cox et al., 2001). Actomyosin contractility regulates cell body contraction (Mitchison and Cramer, 1996) and adhesion turnover regulates cell retraction.

Chemotaxis is an important phenomenon that plays a pivotal role in physiological processes, including embryogenesis/organogenesis, wound healing, leukocyte maturation, lymphoid trafficking and general immunity. The unique pattern of receptor expression and the distinct activity of chemokines allows for the precise control of the above-mentioned functions through tissue-specific leukocyte homing.

Immune Function & Tissue-Specific Leukocyte Homing

Leukocytes express several types of chemokine receptor that are required for extravasation and positioning within secondary lymphoid organs. Receptors are upregulated (CXCR5, CCR4 and CCR8) and downregulated (CCR7) following T cell activation to promote the cooperation with B cells (that secrete MDC (CCR4 ligand) among others) in primary immune responses and to direct these new subsets of cells to specific areas within the body depending upon the nature of the response required (fig.1.9).

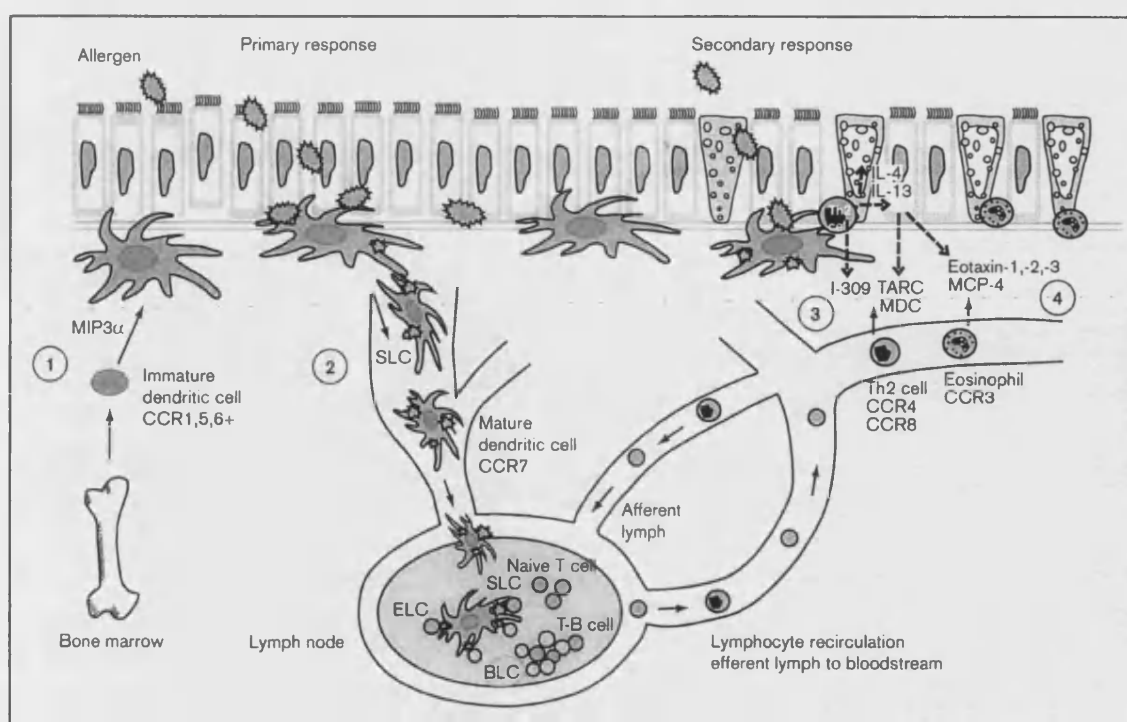


Figure 1.9. The role of chemokines in general allergic pulmonary inflammation. (1) Immature DCs traffic from the bone marrow in to the respiratory epithelium. (2) Once DCs have picked up antigen in the tissue they become activated and downregulate specific chemokine receptors and upregulate CCR7. This results in the DCs migrating to the lymph node where they activate naïve T cells generating Th2 cells. (3) These Th2 cells then leave the lymph node and travel back to the lung where they can be reactivated by exposure to inhaled antigen. Activated Th2 cells release Th2 cytokines (IL-4 and IL-13), which then induce the release of eotaxins (CCL11, CCL24, CCL26) and the CCR4 ligands MDC (CCL22) and TARC (CCL17) from the respiratory epithelium. (4) These ‘Th2 chemokines’ then recruit more Th2 cells as well as other leukocytes (eosinophils, basophils, mast cells). SLC (CCL21), MIP-3 α (CCL20), I-309 (CCL1), ELC (CCL19), BLC (CXCL13), MCP-4 (CCL13). Reproduced from (Luster, 2001).

Immature DCs are attracted to the tissue by inflammatory chemokines and, once in the vicinity of the inflammatory stimulus, pick up antigen causing the DCs to mature in to cells capable of activating lymphocytes. During this maturation DCs downregulate their expression of CXCR1, CCR1, 2, 5, 6 and upregulate CCR7 (and CXCR4/CCR4) expression (Dieu et al., 1998), making the cells less responsive to inflammatory chemokines but allowing them to respond to CCL21 and CCL19 (both CCR7). These guide the cell into the lymphatics and, ultimately, into the T cell rich regions of lymph nodes where they are able to interact with and stimulate T cells (Forster et al., 1999; Sallusto et al., 1999b; Sallusto and Lanzavecchia, 2000). Chemokines are also involved in bringing naïve T and B cells from blood across high endothelial venules (HEV) into the

lymph nodes and in to the T and B cell follicles. CCL19 and CCL21 are required for the T cell localisation, whereas CXCL13 is required for the B cells (Cyster, 1999; Gunn et al., 1998a; Gunn et al., 1998b). This activation of T cells leads to their polarization and the generation of distinctive sets of T cells. Th1 cells express high levels of CCR5 and CXCR3 and are directed towards sites where IFN γ has induced the production of these receptors' respective chemokines. Th1 cells play a role in the defence against intracellular bacteria and many viruses. Th2 cells express CCR4, CCR8 and CCR3, and IL-4/IL-13-induced production of their respective chemokines recruits these cells to sites of allergic inflammation (and parasitic infections) (Moser and Loetscher, 2001). Follicular homing T (Tfh) cells upregulate expression of CXCR5 and are localised to within germinal centres of secondary lymphoid organs and may play a role in antibody responses (Mackay, 2000). All of these T cell subsets downregulate CCR7.

Where trafficking of CCR7+ T lymphocytes is generally restricted to T cell areas of lymphoid organs, effector memory T (T_{EM}) cells are found at low levels in every tissue within the body and are preferentially recruited to extra-lymphoid sites and display selective homing abilities for tissue type. Some of these cells must also mount secondary proliferative responses and therefore require CCR7 expression (and L-selectin – binds E-selectin, MAdCAM and all sLe^x-like sugars) – central memory T (T_{CM}) cells. T_{EM} cells combat the spread of pathogen whereas T_{CM} guard against subsequent infections. Naïve T cells are distinguished from memory by the presence or absence of CD45RA, respectively (memory are CD45RO⁺), with CCR7 expression distinguishing the two types of memory T cells (T_{CM} cells can differentiate in to T_{EM} cells) (Sallusto et al., 1999a). These T_{EM} cells can be further subdivided and these subsets display remarkable specificity for types of tissue. CLA⁺ (cutaneous lymphocyte antigen – binds E-selectin) $\alpha 4\beta 1^+$ (VLA-4 – binds VCAM-1, fibronectin, $\alpha 4$ integrin) CCR4⁺ CCR10⁺ T_{EM} cells migrate specifically to subcutaneous tissue (skin) (Campbell et al., 1999a; Homey et al., 2000); $\alpha 4\beta 7^+$ (binds MAdCAM, fibronectin, VCAM-1 weakly) CCR9⁺ T_{EM} cells migrate specifically to gut mucosal tissue (fig. 1.10) (Zabel et al., 1999). The memory T cells lack L-selectin whereas all other leukocytes express it, and both memory populations possess increased levels of LFA-1 ($\alpha L\beta 2$ selectin – binds ICAM 1-5) (Von Andrian and Mackay, 2000).

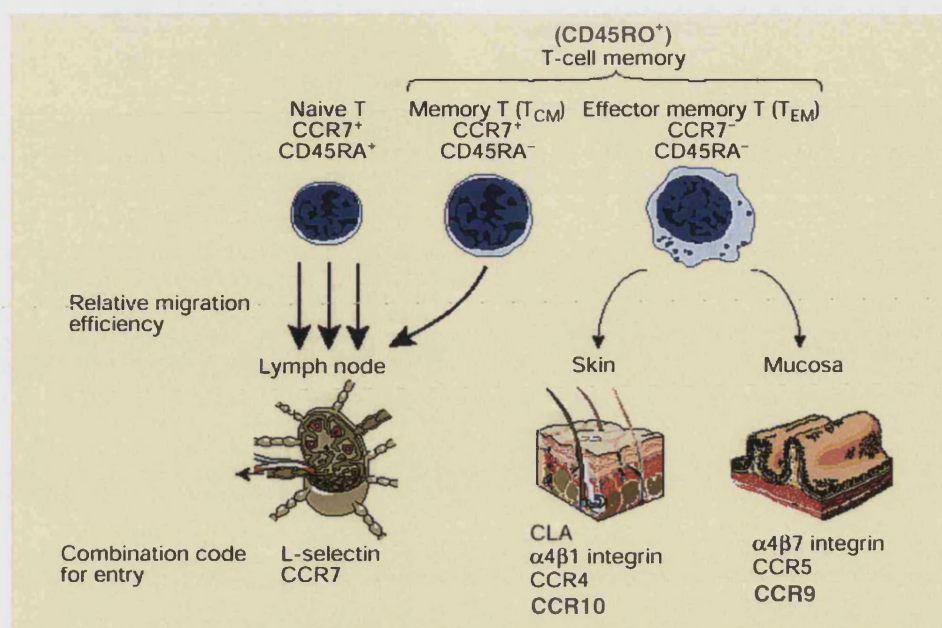


Figure 1.10. The different migration preferences for different types of memory cell. See text for more detail (Mackay, 1999).

Chemokines & Pathology

The ease in which induction of chemokines can occur along with their high levels of expression can, and does, lead to inappropriate tissue damage. The initial chemokine-recruited leukocytes may induce even higher levels of new chemokine expression leading to more extensive tissue damage, a feed-forward mechanism. Therefore, the protective effects of leukocytes can lead to disease. There is widespread evidence of chemokine expression in diseases (table 1.5) and one of the human pathological conditions in which chemokines or their receptors have been unequivocally implicated in is HIV infection.

In the mid-1990s, chemokines got immense publicity when it emerged that CCR5 and CXCR4 were co-receptors for HIV and that their ligands were able to block HIV entry into CD4⁺ cells (macrophages (M-tropic – CCR5) and T cells (T-tropic – CXCR4)) (Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). The importance of these receptors for HIV entry is highlighted by the fact that individuals who lack functional CCR5 are resistant to HIV-1 infection (Samson et al., 1996a). Over the last few years a large number of chemokine receptors have been shown to serve as co-receptors for the virus (Simmons et al., 2000).

Table 1.5. A selection of diseases that chemokines/receptors have been implicated in their pathology (either due to increased or decreased expression on cells or in tissue).

Diseases	Chemokine Receptors Involved
<i>Liver Disease</i>	
Ischeamia reperfusion	CCR2, CCR9/10, CXCR2, CXCR3
Hepatitis	CCR2, CCR9/10, CXCR3, CXCR1, CXCR2
Graft-Versus-Host Disease (GVHD)	CCR1, CCR5, CCR9/10, CXCR3
Liver cancer	CXCR3, CXCR4
<i>Respiratory Disorders</i>	
Asthma/Allergic inflammation of the airways	CCR1, CCR2, CCR3, CCR4, CCR8 CXCR1, CXCR2
Idiopathic pulmonary fibrosis	CXCR1, CXCR2, CXCR3
Chronic bronchitis	CXCR1, CXCR2, CCR2
Chronic obstructive pulmonary disease	CXCR1, CXCR2, CCR2
<i>Autoimmune Diseases</i>	
Multiple sclerosis/EAE	CXCR3, CCR2, CCR5
Type-1 diabetes	CXCR3
GVHD	CXCR3, CCR1, CCR2, CCR5
Rheumatoid arthritis	CCR2, CCR5, CXCR2, CXCR3
<i>Neoplasia</i>	
Tumour growth	CXCR1, CXCR2, CXCR3, XCR1,
Metastasis formation	CXCR4, CCR7, CCR10
<i>Infections</i>	
HIV	Principally CXCR4 and CCR5
Poxvirus	CXCR3
Endotoxic shock/Sepsis	CCR4
<i>Vascular Diseases</i>	
Atherosclerosis	CCR2, CXCR2
<i>Miscellaneous</i>	
Wound Healing	CXCR1, CXCR2
Psoriasis	CXCR2, CCR4, CCR6, CCR10

An interesting structural feature of the CXC chemokines with relation to diseases is the presence of the ELR motif preceding the first cysteine in the CXCR1 and CXCR2 receptor ligands. These chemokines have been shown to be angiogenic whereas the ELR-negative CXC receptor ligands are angiostatic. Therefore, the angiogenic (promote new blood vessel formation) chemokines have been implicated in tumours, where a good blood supply is essential, and metastasis. Conversely, angiostatic chemokines inhibit tumour growth. Other diseases associated with angiogenesis include psoriasis, rheumatoid arthritis (pannus formation) and idiopathic pulmonary fibrosis. These references provide far more in depth

information on the roles of chemokines and their receptors in disease (Baggiolini, 2001; Gerard and Rollins, 2001; Homey et al., 2002; Murphy et al., 2000; Owens et al., 2001; Rossi and Zlotnik, 2000).

Chemokines & Their Receptors as Therapeutic Targets

To date there are currently no chemokine receptor antagonists or agonists on the market to treat a pathological condition. However, the development of knockout and transgenic mice for almost all the chemokine receptors and many of the chemokines has demonstrated the importance of chemokines in disease (and physiology) and the potential benefit as therapeutic targets (table 1.6). In general, the deletion of chemokines and receptors pivotal to basal trafficking and homing have noticeable phenotypes, whereas those that are inducible are viable and normal, with phenotypic differences only evident when the animals are exposed to specific inflammatory conditions (although there are differences in leukocyte compartmentalisation in the absence of stimuli). To date, only the CXCR4 knockout has proven to be embryonically lethal. There is extensive research being carried out to look at the potential role of chemokine receptor agonists for diseases such as HIV and conditions like cancer, and the role of antagonists in chronic deleterious immune conditions (Cascieri and Springer, 2000). Three types of antagonist stand out: small molecule chemokine receptor inhibitors (e.g. TAK-779 a CCR5 antagonist (Baba et al., 1999)); modified chemokines e.g. MetRANTES (arthritis/airway inflammation) and AOP-RANTES (HIV-1 infection) – both N-terminal peptides (Plater-Zyberk et al., 1997); and monoclonal antibodies to chemokine receptors or ligands (e.g. anti-Mig (Koga et al., 1999)). A recently found twist in the tail is the ability of CXCR3 agonists to act as CCR3 antagonists (Loetscher et al., 2001), and the CCR3 agonists, CCL11/CCL26, to inhibit CCR2 (Ogilvie et al., 2001; Ogilvie et al., 2003), suggesting a new mechanism of regulation of leukocyte recruitment during inflammatory and immune reactions. The next few years is likely to see a flurry of chemokine-related treatments for various diseases entering clinical trials and whether the treatments currently in Phase I clinical trials are able to make it to the market (Boehncke and Schon, 2003; Gao and Metz, 2003; Houshmand and Zlotnik, 2003; Onuffer and Horuk, 2002; Proudfoot et al., 2003; Schwarz and Wells, 2002).

Table 1.6. Chemokine receptor knockouts and transgenic models.

CHEMOKINE RECEPTOR KNOCKOUTS	
<i>CC Receptors</i>	
CCR1	CCR1 knockout mice have defective NK, neutrophil and myeloid progenitor cell trafficking; they are more susceptible to <i>Aspergillus fumigatus</i> and <i>T. gondii</i> infection, and nephrotoxic nephritis, but less susceptible to Schistosome egg granuloma formation in the lung, pancreatitis-associated ARDS, EAE, and acute and chronic cardiac allograft rejection. Mice have an imbalance in Th1/Th2 cytokines and impaired granulomatous inflammation.
CCR2	Reduced atherosclerosis in ApoE ^{-/-} mice, at least partly due to defects in macrophage recruitment; Imbalance in the production of Th2 cytokines, such as IFN γ ; Increased susceptibility to pulmonary <i>Cryptococcus</i> infection; Reduced Langerhans cell migration to draining lymph nodes; Increased severity of experimental glomerulonephritis; Inability to clear <i>Listeria</i> infections but less susceptible to <i>L. donovani</i> infection, cockroach allergen-induced bronchial hyperreactivity, and granuloma formation (beta glucan, Schistosome egg or PPD challenge), but were more susceptible to the injurious effects of <i>Aspergillus fumigatus</i> challenge in the lung; Reduced EAE susceptibility (MOG model); Partial protection in DSS-induced colitis; CCR2 is a negative regulator of hematopoiesis.
CCR3	No effect on the basal trafficking of eosinophils to the lung but does effect trafficking to the intestinal mucosa. However, eosinophil recruitment to the lung is severely curtailed following antigen challenge, but paradoxically, an increase in airway hyperresponsiveness to methacholine stimulation and increased mast cell accumulation to the airways. Additionally, knockout mice showed reduced infiltration of eosinophils to the skin in an atopic dermatitis model, with no effect on Th2 or mast cell recruitment.
CCR4	Increased resistance to endotoxic shock (LPS-induced), but no effect on a classical model of airway inflammation. However, there is an attenuation of airway hyperresponsiveness in an <i>Aspergillus</i> model of chronic inflammation of the airways. Involved in the recruitment of cutaneous memory T cells to the skin but only in concert with CCR10.
CCR5	Increased mortality following <i>Cryptococcus</i> infection; Reduced clearance of <i>Listeria</i> infections; No protection in EAE; Resistance to DSS-induced colitis; Enhanced T cell-dependent immune responses (DTH reaction); Reduced macrophage recruitment and demyelination in a mouse model of hepatitis intracranial infection, but comparable T cell migration to wild-type mice; Protection against various M-tropic strains of HIV.
CCR6	Reduced humoral immune response to oral antigens and to enteropathic rotavirus but systemic response are normally in response to subcutaneous injection of antigen; Increased T cell subsets within the mucosa; Enhanced inflammation in a contact hypersensitivity model; No inflammation in a DTH model; Reduced airways resistance, eosinophilia around the airways in a cockroach antigen model of allergic inflammation of the airways.
CCR7	Altered secondary lymphoid organ structure due to impaired lymphocyte migration, with DCs failing to migrate to draining lymph nodes upon activation; Reduced humoral immune responses (DTH) and contact sensitivity.
CCR8	Defective Th2 responses in models of Schistosoma mansonii-soluble egg antigen-induced granuloma formation and in ovalbumin and cockroach antigen-induced airways inflammation, with reduced eosinophilia; Normal Th2 development but aberrant Th2 cytokine production; Another asthma model demonstrated no effect of CCR8 deletion on disease progression.
CCR9	No effect on intrathymic development of T cells but there were fewer pre-pro B cells. However, this had no effect on the generation of a normal complement of mature B cells; Reduced intraepithelial T cell to epithelial cell ratio.
CCR10	No data available.
CCR11	No data available.

Table continued on the following page ►

CXC Receptors	
CXCR1	Reduced neutrophil recruitment.
CXCR2	Lymphadenopathy, splenomegaly and increased B cell numbers; Delayed wound healing; Defective acute neutrophil accumulation; Reduced macrophage recruitment in atherosclerosis; Required for protection against <i>Toxoplasma gondii</i> infection and urinary tract infection; Protection against septic injury.
CXCR3	Resistance to GVHD (cardiac); Phenotypically normal.
CXCR4	Non-viable embryos; Defective vascular development, haematopoiesis, cardiogenesis and derailed cerebellar neuronal migration; Defective T cell development due to the lack of localisation of early lymphoid progenitors to tissue regions of the thymus.
CXCR5	Defects in B cell homing and defective lymph nodes, with abnormal germinal centre formation in the spleen and lack of Peyer's patches.
CXCR6	No data available.
Others	
CX3CR1	Decreased atherosclerosis; Selective reduction in NK cells; Phenotypically normal; No effect in animal models of EAE and glomerulonephritis; May have a role to play in cardiac GVHD; Role of CX3CR1 on DCs, monocytes and NKs still unclear as recruitment unaffected in a murine model of peritonitis.
XCR1	No data available.
Chemokines	
MCP-1/CCL2	Reduced atherosclerosis in ApoE ^{-/-} mice; Protection in EAE.
MIP-1 α /CCL3	Reduced inflammation of the airways; No protection in EAE.
Eotaxin/CCL11	No effect on eosinophil accumulation in lung inflammation; Eotaxin gene knockout and protein neutralization in animal models has suggested a role for CCR3 in eosinophil mobilisation and distribution (intestinal but not lung), and in allergic inflammation (skin); An alternative study demonstrated 70% reduction in eosinophil recruitment to the airways in the ovalbumin model of allergic inflammation of the airways.
IP-10/CXCL10	Prolonged survival of hearts in GVHD model; No effect on the trafficking of T cells to the CNS in an EAE model.
SDF-1/CXCL12	Similar phenotype to its receptor (CXCR4) knockout.
TRANSGENIC EXPRESSION OF LIGANDS	
MCP-1/CCL2	Exacerbation of ischemic brain injury, with recruitment of inflammatory cells; Transient and severe encephalopathy.
MIP-1 α /CCL3	Increased neutrophil and NK cell accumulation and stimulation of innate immunity in murine bacterial pneumonia.
TECK/CCL25	Localisation of activated CD8 $\alpha\beta$ lymphocytes to intestinal mucosa.
IP-10/CXCL10	Increased leukocyte infiltration in the CNS; Increased airway hyperreactivity and airway inflammation in mouse model of asthma
SDF-1/CXCL12	Enhanced myeloid progenitor cell survival/antiapoptosis and myelopoiesis (marrow and splenic); Recruitment of DCs, B cells and plasma cells (but not T cells) to pancreatic islets after ectopic expression of SDF-1/CXCL12.
BLC/CXCL13	In addition to attracting B cells, BLC/CXCL13 expression in pancreatic islets led to development of lymph node-like structures that contained B and T cell zones, high endothelial venules, stromal cells, and the chemokine SLC/CCL21 (lymphoid neogenesis).

The chemokine receptors have been colour coded to denote homeostatic, homeostatic/inflammatory, or inflammatory (no colour) chemokine receptors. Please note that the chemokine knockout and transgenic section is by no means an exhaustive collection of the published data. The majority of the data is derived from the following references: (Lucas and Greaves, 2001; Power, 2003; Proudfoot, 2002; Proudfoot et al., 2003).

1.4 CC Chemokine Receptor 4 (CCR4)

The research contained within this thesis is primarily concerned with the signal transduction pathways downstream of CCR4 upon ligation with its respective ligands, MDC/CCL22 and TARC/CCL17. For that reason, a brief review of CCR4/MDC/TARC biology follows.

Physiological Characteristics of CCR4, MDC & TARC

CCR4

CCR4 is a 41.4 kDa seven-transmembrane G protein-coupled receptor with a high affinity for the ligands, Thymus- and Activation-Regulated Chemokine (TARC/CCL17) and Macrophage-Derived Chemokine (MDC/CCL22) (Imai et al., 1996; Imai et al., 1998). Previously known as K5-5, it has an open reading frame predicting a polypeptide 360 amino acids long with the gene mapped to chromosome 3p24 (Samson et al., 1996b). The cDNA was originally cloned from a human basophilic leukaemia cell line in 1995, and RANTES/CCL5, MCP-1/CCL2 and MIP-1 α /CCL3 have shown to be weak CCR4 agonists (Power et al., 1995b).

CCR4 is expressed on T helper cells type 2 (Th2s) (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Sallusto et al., 1998), CLA⁺ cutaneous memory T cells (Campbell et al., 1999a), natural killer (NK) cells (Godiska et al., 1997; Innngjerdigen et al., 2000), thymocytes (Andrew et al., 2001; Chantry et al., 1999; Taylor, Jr. et al., 2001), immature dendritic cells, basophils, monocytes and platelets (Abi-Younes et al., 2001; Clemetson et al., 2000; Power et al., 1995a; Power et al., 1995b). CCR4 functions currently include the migration of CLA⁺ cutaneous memory T cells to the skin (Campbell et al., 1999a; Campbell and Butcher, 2000); dendritic cell trafficking (Godiska et al., 1997); T cell maturation (Campbell et al., 1999b; Chantry et al., 1999); and Th2 cell recruitment to areas of antigen (Imai et al., 1999).

TARC

TARC is an 8 kDa (71 amino acids (94 amino acids (ORF) with first 23 forming a signal peptide)) secreted protein that binds with high affinity ($K_d < 0.5$ nM) to CCR4 (Imai et al., 1996; Imai et al., 1997) and has also been linked to CCR8 (fig. 1.11) (Bernardini et al., 1998). It is constitutively expressed by the thymus, monocytes and dendritic cells and can be induced in other cells, such as B cells, keratinocytes and airway epithelial cells, but not T cells (Hashimoto et al., 1999; Imai et al., 1999).

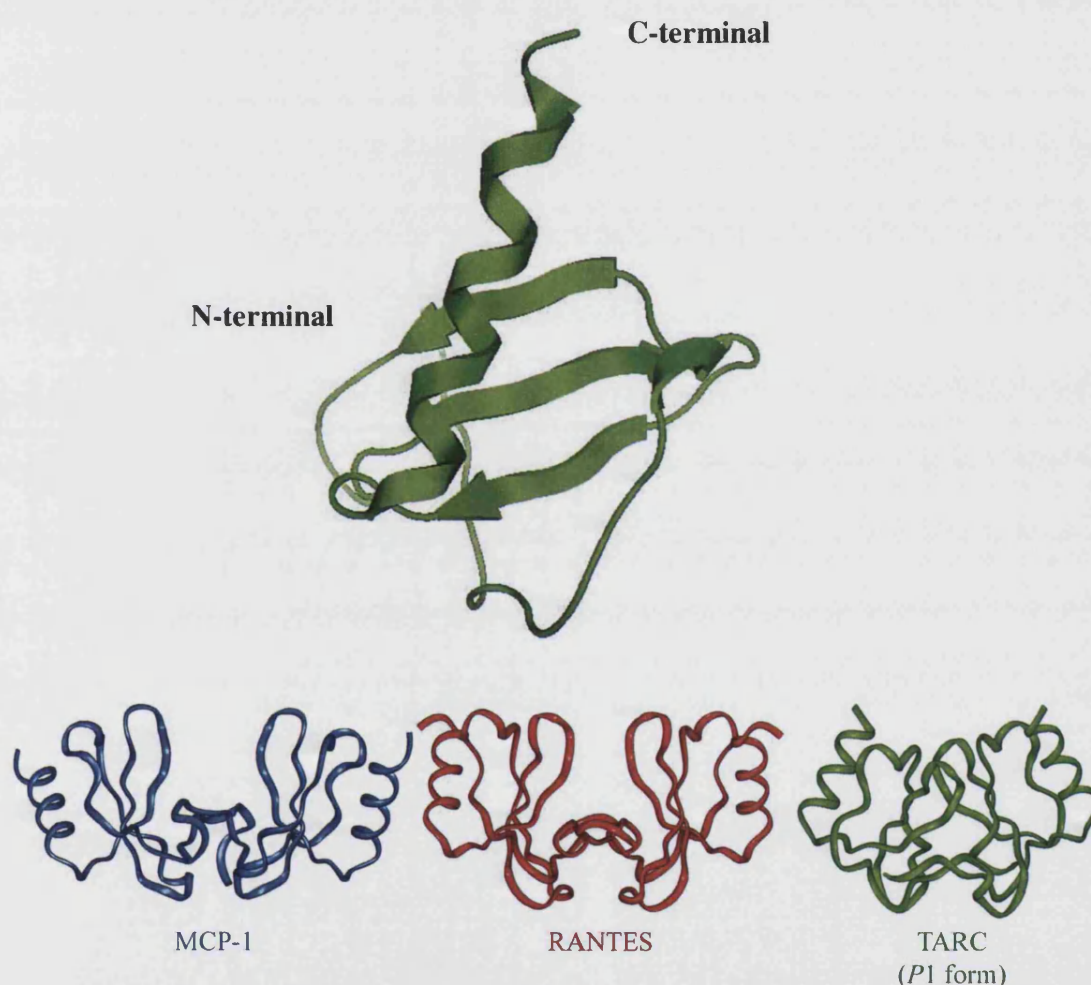


Figure 1.11. Structure of TARC. Each monomer of TARC has the characteristic fold of CC chemokines consisting of a three-stranded anti-parallel β -sheet flanked by a C-terminal α -helix (top). In the majority of CC chemokines, dimerization is promoted by interactions between the N-termini of both monomers, while the C-terminal helices are the most distant regions of the molecules. Although both of these features are present in the case of TARC, the dimers found are more compact compared with other CC chemokines. Reproduced from (Asojo et al., 2003).

MDC

MDC, like TARC, is also an 8 kDa (69 amino acids (93 amino acids ORF)) secreted protein with high affinity ($K_d \approx 0.2$ nM) exclusively for CCR4 (Godiska et al., 1997). It is constitutively expressed by macrophages, mature dendritic cells, intestinal epithelial cells and B cells, and can also be upregulated in monocytes and T cells (Andrew et al., 1998; Berin et al., 2001a; Galli et al., 2000; Vulcano et al., 2001). Both MDC and TARC are classed as homeostatic and inflammatory chemokines (fig. 1.5) and share a 32% sequence similarity (Mantovani et al., 2000).

Pathological Roles of CCR4, MDC & TARC

Figure 1.12 shows the pathological conditions that CCR4 and/or its ligands have been implicated in. Until recently, there has been a lack of neutralising monoclonal antibodies, small molecule antagonists and knockout mice towards CCR4, which has made it hard to determine the role of the receptor in pathologies. Consequently, potential roles for CCR4 in diseases have been largely inferred based on the analysis of TARC and MDC in animal models of diseases.

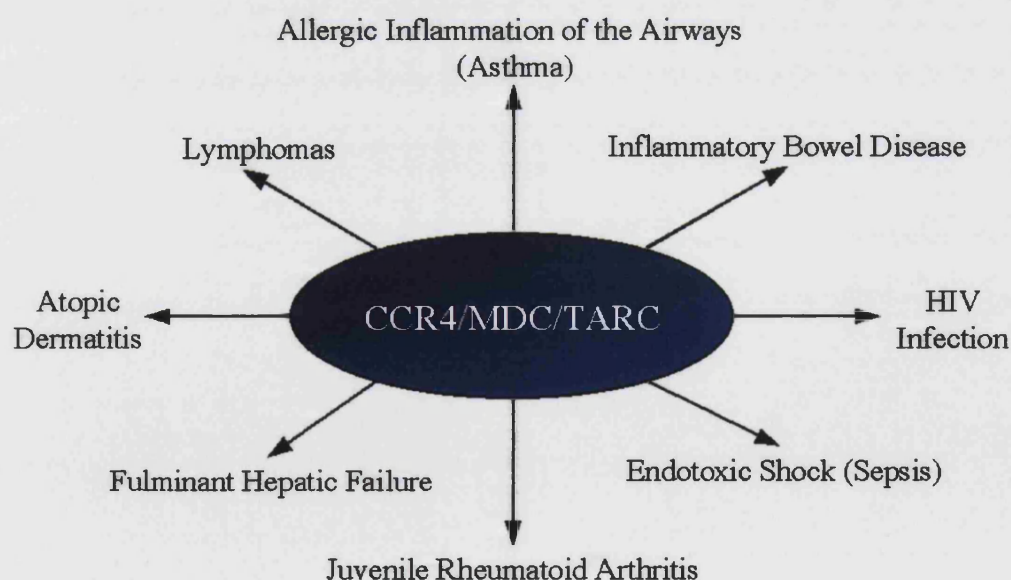


Figure 1.12. A few of the pathological conditions CCR4 and its ligands have been implicated to play a role.

Airway Disease

Owing to the initial belief that CCR4 was exclusively expressed on Th2 cells, early pathological roles for CCR4 concentrated on known Th2-mediated conditions. One of the most widespread Th2-mediated diseases in the general population, and consequently most profitable should a first-rate treatment be found, is allergic inflammation of the airways (e.g. asthma). As a result, the majority of research into the role of CCR4 in diseases has been in this area. A study by Lloyd et al. (Lloyd et al., 2000), showed that MDC/CCR4 plays a critical role in the homing of antigen-specific Th2 cells in a T cell transfer model of allergen-induced lung injury in mice. This migration of Th2 cells gave rise to eosinophilia and bronchial hyperresponsiveness. The CCR3/CCL11 pathway is critical in the acute stages but CCR4/MDC dominates the chronic inflammatory response (after repeated allergen challenge), being primarily responsible for the long-term recruitment of antigen specific Th2 cells to the airways. It has been shown that the bronchial epithelial cells are a source of TARC, with more intense expression in asthmatics compared to normal subjects (Berin et al., 2001b; Panina-Bordignon et al., 2001; Sekiya et al., 2000). A recent study has demonstrated that bronchial epithelial cells are also a source of MDC, and that the BAL fluid of asthmatics has massively increased levels of MDC compared to normals (Panina-Bordignon also showed MDC levels increased but Sekiya found MDC levels were unaltered in asthmatics compared to normal) (Bochner et al., 2003; Lezcano-Meza et al., 2003). TNF α + IL-4 and IFN γ separately were able to induce the expression of TARC and, with all three, the enhancement was greatest, and showed that IL-4 on its own has no effect both *in vitro* and *in vivo* (Berin et al., 2001b). In addition, naïve T cells have been observed to produce both TARC and MDC, contributing to the local migration of Th2 cells (Hirata et al., 2003). Interestingly, glucocorticoids were shown to almost completely inhibit TARC expression at both the mRNA and protein levels, suggesting that the beneficial effects of glucocorticoids in current treatments for asthma may be at least in part due to their inhibitory effects on TARC production by the bronchial epithelium. Steroidal treatment has also been demonstrated to shift the balance away from CCR4 Th2 cells to CXCR3 expressing T cells (Kurashima et al., 2001). Recently, a group showed for the first time that TARC was pivotal in the development of Th2-mediated allergen-induced asthma, with eosinophilia and airway hyperresponsiveness, by attenuating this condition with an antibody against TARC. It was also the first time that there had been success in controlling

Th2 cytokine production *in vivo* by targeting a chemokine (Kawasaki et al., 2001). Mouse polyclonal antibodies against MDC have also been shown to protect against eosinophilia and bronchial hyperresponsiveness despite MDC not appearing to play a role in inflammatory airway disease according to other groups (Gonzalo et al., 1999). In patients with chronic hypereosinophilia, high levels of TARC (but not MDC) induces the down-regulation of CCR4 on Th2 cells *in vivo*, and that the source of TARC is likely to be dendritic cells stimulated by Th2 cytokines. This receptor down-regulation can be inhibited with a mAb against TARC (but not MDC) (de Lavareille et al., 2001). Panina-Bordignon and co. (Panina-Bordignon et al., 2001) demonstrated there was a significantly higher number of infiltrating CCR4⁺ Th2 cells compared with controls, but Campbell et al. (Campbell et al., 2001) showed that there was no difference in the expression of receptors on T cells in asthmatic subjects compared with normals. They did observe that the lung T cell expressed a profile of chemokine and adhesion receptors distinct from gut or skin homing T cells. Another study that quenches the role of CCR4 in asthma much more seriously used a knockout model. The deletion had no effect on Th2 differentiation *in vitro* or in a Th2-dependent model of airway inflammation, with no differences in eosinophilia or bronchial hyperresponsiveness (Chvatchko et al., 2000). In contrast, a second CCR4^{-/-} model displayed attenuated airway hyperresponsiveness in an *Aspergillus fumigatus* mouse model of airway inflammation (there was no effect on airway remodelling) (Schuh et al., 2002). Recently, a study using an anti-CCR4 Ab had no effect on a guinea-pig model of allergic inflammation of the airways (Conroy et al., 2003). These conflicting views on the role of CCR4 in asthma may be explained by the intrinsic differences in blocking with a ligand rather than a receptor; using genetically modified mice rather than a neutralising antibody; or the varying model system. Two other airway diseases, chronic obstructive pulmonary disease and pulmonary sarcoidosis, are characterised by infiltrating T cells that lack CCR4 (Panina-Bordignon et al., 2001).

Skin Disease

Another extensive area of CCR4-mediated disease research is that of atopic dermatitis. Four years ago it was observed that CCR4 is present on almost all cutaneous lymphocyte antigen (CLA) expressing memory T cells and other systemic memory T cells that are CLA⁺ and $\alpha 4\beta 7^{+}$ (gut homing T cells are $\alpha 4\beta 7^{+}$) (Campbell et al., 1999a). Major subsets of

these memory T cells have both Th1 and Th2 potential expressing the receptor CCR4 as well as the Th1-associated receptor CXCR3 (Andrew et al., 2001). The CLA⁺ memory T cells are skin homing and have been found in areas of chronically inflamed skin, and TARC induces the integrin-dependent adhesion of these cells to ICAM-1 (Campbell et al., 1999a). A paper published the same year showed that TARC is highly expressed in a mouse model of atopic dermatitis and not at all in controls, and that keratinocytes could be induced to produce TARC. MDC levels were also upregulated and the source identified as dermal dendritic cells (Vestergaard et al., 1999) ((Vestergaard et al., 2000) showed that the same features occurred in the human form of the disease as in mice). Additionally, TARC production by maturing Langerhans cells (immature DCs found in epidermis) has been observed in response to IL-4 (Xiao et al., 2003). Subsequently, a relatively large number of papers have confirmed the role of CCR4 and its ligands in atopic dermatitis (Biedermann et al., 2002; Okazaki et al., 2002; Uchida et al., 2002; Vestergaard et al., 2003; Zheng et al., 2003). Another pathological condition concerning the skin, psoriasis vulgaris, has implicated CCR4 in the T lymphocyte homing to the psoriatic dermis along with CXCR3 (Rottman et al., 2001). There appears to be a role for CCR4-expressing Tc2 cells (Inaoki et al., 2003) in this condition, but this is generally considered to be a Th1 disease and other groups have demonstrated no role for CCR4-expressing skin-homing cells (Vestergaard et al., 2003).

Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a Th1-mediated disease and, as mentioned above, CCR4 is not present on $\alpha 4\beta 7^+$ (gut homing) T cells (<10%). However, MDC and TARC levels are increased in animal models of colitis along with CCR4 (Scheerens et al., 2001). A population of circulating T cells that are CD45RO⁺ $\beta 7^{\text{hi}}$ and are thought to preferentially migrate to the intestinal lamina propria and epithelium are CCR4⁺ (28%) (Agace et al., 2000). Conversely, a recent study indicated that CCR4 expressing memory T cells had a role to play in Crohn's disease but not ulcerative colitis (Jo et al., 2003). Furthermore, MDC has been shown to be constitutively expressed and upregulated on intestinal epithelial cells (Berin et al., 2001a).

Miscellaneous Diseases

Other diseases CCR4 and/or MDC/TARC have been implicated in include endotoxic shock (Chvatchko et al., 2000); rheumatoid and juvenile rheumatoid arthritis (Katschke, Jr. et al., 2001; Ruth et al., 2001; Thompson et al., 2001); anti-leukaemia T cells (Ghia et al., 2001); Fulminant hepatic failure (Yoneyama et al., 1998); Hodgkin's lymphoma (van den et al., 1999); and T cell non-Hodgkin lymphoma (Jones et al., 2000); Adult T cell lymphoma (Ishida et al., 2003; Yoshie et al., 2002). Recently, a study observed that CCR4-expressing regulatory T cells appeared responsible for the reversal of Type I diabetes upon treatment with an anti-CD3 mAb (Herold and Taylor, 2003; Kim et al., 2002). Finally, MDC exhibits antiviral activity against M- and T-tropic HIV-1 strains, however, this does not occur via CCR4 (Struyf et al., 1998). Moreover, MDC is able to induce human eosinophil chemotaxis despite the lack of CCR4 mRNA (Bochner et al., 1999; Borchers et al., 2002; Nagase et al., 2001). Therefore, there appears to be a second unidentified receptor for MDC and this may explain some of the conflicting results observed in other disease models.

1.5 Signal Transduction Pathways

Overview

Thus far, the pathophysiology of chemokines and their receptors have been discussed. A chemokine exerts its effect on a cell by activating a plethora of signal transduction pathways via ligation of its specific receptor (e.g. CCR4). This leads to the induction of a cellular function, such as cell spreading, proliferation, cytokine secretion, and cell migration. The following section will review the principal players and the signalling pathways thought to be involved in chemokine-mediated directed cell migration.

As with all G-protein coupled 7-transmembrane receptors, the receptor acts as a ligand-dependent GDP exchange factor with receptor ligation leading to the exchange of GDP for GTP in the receptor-associated GDP-bound G protein. This leads to the G_α and $G_{\beta\gamma}$ subunits dissociating and activating various signalling cascades, with the receptor reverting to a low affinity state. Subsequently, following GTP hydrolysis, the G_α subunit (now in its GDP-

bound inactive form) reassociates with the $G_{\beta\gamma}$ subunit and terminates the signalling response. To date, almost all chemokine receptor activated signalling pathways are *Bordetella pertussis* toxin (PTX) sensitive, indicating that the $G_{\alpha i}$ subunit is the principal $G\alpha$ protein utilised (PTX catalyses ADP-ribosylation of the $G_{\alpha i}$ protein thus preventing it interacting with the receptor). However, some chemokine receptors also demonstrate the ability to couple to other G-proteins as well, such as $G_{\alpha q}$, $G_{\alpha 16}$ and $G_{\alpha 11}$ (Kuang et al., 1996; Soede et al., 2001). Nevertheless, only $G_{\alpha i}$ coupled 7TMR display chemotactic activity.

Phospholipase C (PLC)

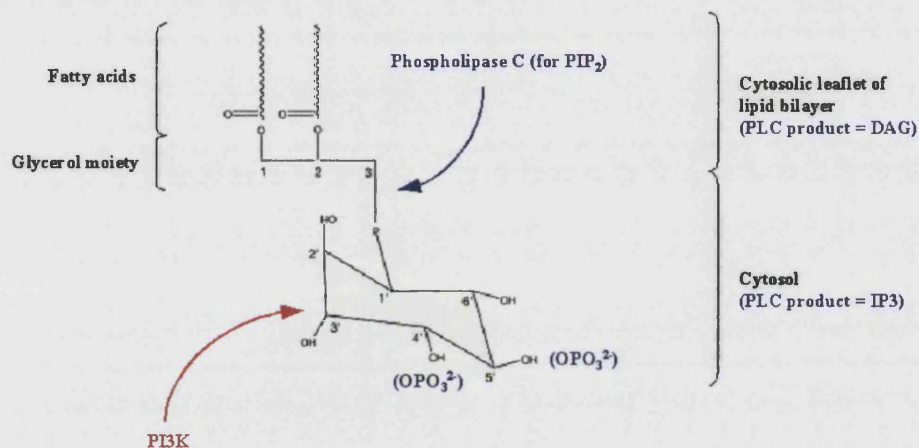


Figure 1.13. Phosphatidylinositol structure (PI – structure represented in black). PI is the basic building block for the intracellular inositol-containing lipids, which consist of a D-myo-inositol-1-phosphate (Ins 1-P) linked via its phosphate group to diacylglycerol (DAG). Phospholipase C (PLC) hydrolyses PI(4,5)P₂ (PIP₂) to generate inositol (1,4,5)-trisphosphate and DAG (required substrate alterations to PI (i.e. structure of PIP₂) highlighted in blue). The inositol head of PI has five free hydroxyl groups but only groups at positions 3, 4 and 5 can be phosphorylated in different combinations, with phosphatidylinositol 3-kinase acting at the 3 position (highlighted in red). Diagram modified from (Sotsios and Ward, 2000).

The phosphoinositide-specific phospholipase C (PLC) isozymes play a key role in signal transduction by hydrolysing the membrane phospholipid phosphatidylinositol 4, 5-bisphosphate (PI(4,5)P₂), generating the two intracellular second messengers inositol 1, 4, 5-trisphosphate (Ins(1,4,5)P₃ or IP₃) and diacylglycerol (DAG) (fig. 1.13) (Rebecchi and Pentylala, 2000). IP₃ can then act on its receptor present on endoplasmic reticulum to release calcium that then has the potential to activate a number of downstream molecules (refer to “Calcium Signalling” section for more information). The other product generated

from the hydrolysis reaction, DAG, activates members of the protein kinase C (PKC) superfamily among other target proteins. This hydrolysis by PLC is one of the earliest signalling events to occur after receptor ligation. Currently, there are five PLC families (β - 4 isoforms; γ - 2 isoforms; δ - 4 isoforms; ϵ and ζ). Although all the PLCs have multiple cellular regulators, only the PLC β and ϵ isoforms are regulated by heterotrimeric G proteins (Rhee and Bae, 1997; Wing et al., 2001). PLC γ isoforms tend to be regulated by receptor and non-receptor tyrosine kinases, and PLC δ by calcium (Allen et al., 1997). The chemokine receptor-associated $G_{\beta\gamma}$ subunits activate the PLC β_2 and PLC β_3 isoenzymes (Jiang et al., 1997; Li et al., 2000). The $G_{\alpha i}$ subunit is unable to activate the PLC β isoforms directly (Wu et al., 1992). Interestingly, the PLC δ isoform may serve to amplify the response of the PLC β isozymes (fig. 1.14). Calcium binding to the C2 domain of PLC δ increases its enzymatic activity suggesting that calcium may regulate PLC δ and that it is activated secondarily to an increase in intracellular calcium induced by PLC β or PLC γ (Kim et al., 1999b). For more in depth reviews on all the PLC isoforms see (Philip et al., 2002; Rebecchi and Pentylala, 2000).

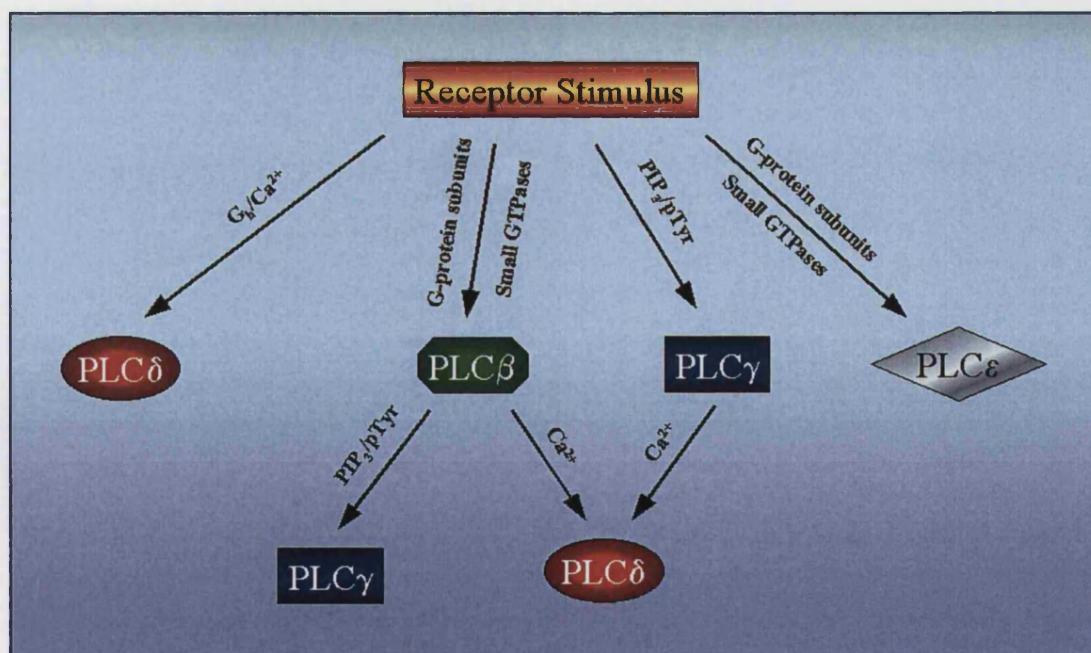


Figure 1.14. Hierarchy of PLC subtypes in cell signalling. A range of receptor stimuli appears to engage the activities of various PLC isoforms with PLC γ and PLC δ additionally playing a possible role in amplifying the initial PLC β response. Diagram adapted from (Rebecchi and Pentylala, 2000).

Calcium (Ca²⁺) Signalling

In London 120 years ago, Sidney Ringer (Ringer, S. (1883) *J. Physiol.* **4**, 29-43) made a landmark observation. He was studying the contraction of isolated rat hearts and noticed that when he switched from suspending the organs in London tap water (which is hard) to distilled water, the beautiful contractions he had observed previously became slower and, after about 20 minutes, the heart would stop beating all together. In order to maintain the contraction he discovered that it was necessary to supplement the suspension medium with calcium salts. Thus, Ringer had dispelled the myth that calcium was exclusively a structural element required for bone and teeth formation and serendipitously revealed that calcium formed part of the signal required for heart contraction (Carafoli, 2002). After a very slow start (calcium's signalling role was not fully appreciated until the late 1950s), calcium has been shown to be involved in many intracellular signalling processes (e.g. gene transcription, proliferation, apoptosis, migration and muscle contraction) and its intracellular mobilisation/accumulation is initiated by a wide variety of receptors.

Resting cells have a cytoplasmic calcium concentration of 100 nM with levels rising to about 1000 nM when the cell mobilises calcium upon activation. The levels of calcium are determined by a balance between the 'on' (calcium into cytoplasm) and 'off' (calcium removed) mechanisms. Cells generate their calcium signals by utilising both internal (release) and external (entry) sources of calcium (Berridge et al., 2000).

The 'ON' Signals

A major intracellular source of calcium is found within the membrane systems of the endoplasmic reticulum (ER), or sarcoplasmic reticulum (SR) of muscle cells. There are two major mechanisms for the release of calcium from these stores and both are via multimeric ligand gated ion channels: IP3 receptor (IP3R) and the ryanodine receptor (RR) (fig. 1.15). The IP3R is operated by IP3, as its name suggests, whilst the RR receptor (named according to its ability to bind ryanodine) is gated by calcium itself (also known as calcium-induced calcium release (CICR)). The RR is, however, also operated by an endogenous ligand named cyclic ADP ribose (cADPR) (Galione and Churchill, 2000), and

both channels are sensitive to physiological changes in calcium (Bootman et al., 2002). Thus, both channels can be considered to be ligand-gated, calcium modulated release mechanisms. There are currently three IP3Rs and three RRs (Berridge et al., 2003). A further internal store operated by nicotinic acid adenine dinucleotide phosphate (NAADP) has been recently identified. The NAADP-mediated release mechanism is insensitive to cytosolic changes in calcium levels and therefore does not support CICR (see reviews (Genazzani and Billington, 2002; Patel et al., 2001)).

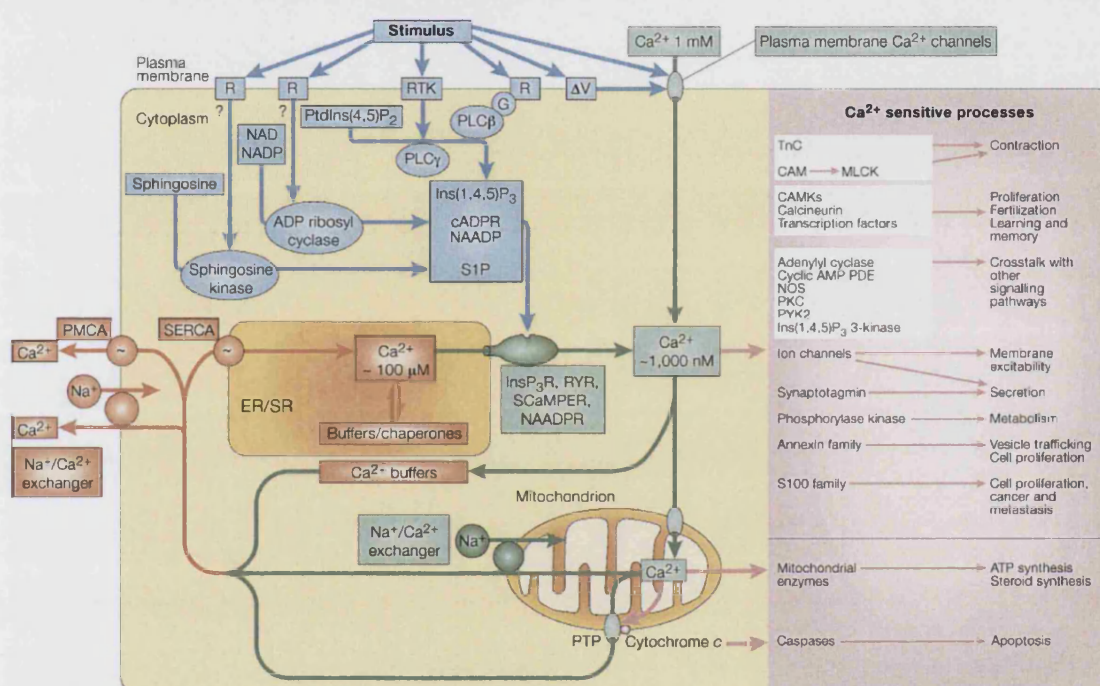


Figure 1.15. An overview of the pathways involved in calcium mobilisation. Calcium mobilising signals are shown in blue. ON mechanisms are in green, with OFF pathways highlighted in red. *Abbreviations:* cADPR, cyclic adenosine diphosphate ribose; CAM, calmodulin; CAMK, calcium/calmodulin-dependent protein kinase; ER, endoplasmic reticulum; G, G-protein; InsP₃R, inositol (1,4,5)-trisphosphate receptor; MLCK, myosin light chain kinase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; NOS, nitric oxide synthase; PDE, phosphodiesterase; PKC, protein kinase C; PLC, phospholipase C; PMCA, plasma membrane calcium ATPase; PTP, permeability transition pore; PYK2, proline-rich tyrosine kinase 2; R, receptor; RTK, receptor tyrosine kinase; RYR, ryanodine receptor; S1P, sphingosine 1-phosphate; SCaMPER, sphingolipid calcium release-mediating protein of the ER; SERCA, sarco(endo)plasmic reticulum calcium ATPase; SR, sarcoplasmic reticulum; TnC, troponin C. Acquired from (Berridge et al., 2000).

Additionally, calcium is able to enter the cell from an external source via various entry channels that vary greatly in their properties: voltage-operated channels (VOCs) are plasma membrane ion channels that are activated by membrane depolarisation; receptor-operated channels (ROCs) open in response to the binding of an extracellular ligand; second-messenger-operated channels (SMOCs) open in response to the binding of intracellular second messengers such as DAG, cyclic nucleotides or arachidonic acid; and store-operated channels (SOCs) open in response to the depletion of internal stores of calcium.

The 'OFF' Signals

For a cell to be able to maintain viability, the calcium influx must be balanced by calcium efflux. Four different mechanisms are responsible – plasma membrane calcium ATPase (PMCA), sodium/calcium exchanger (NCX), sarco(endo)plasmic reticulum calcium ATPase (SERCA), and the mitochondrial uniporter (fig. 1.16). PMCA and SERCA have high affinities but limited capacities meaning they can respond to modest increases in calcium levels and set basal calcium levels. The remaining two calcium transporters, NCX and the mitochondrial uniporter, have much higher capacities and can limit the calcium transient over a wider range (i.e. optimal mitochondrial calcium accumulation occurs when calcium is in the μM range but do accumulate calcium even when presented with modest nM global calcium changes). The PMCA couples ATP hydrolysis to the transport of calcium from cytosolic to extracellular spaces. NCX (plasma membrane) exchanges three moles of sodium for one mole of calcium, either inward or outward, depending on the ionic gradients across the membrane. The mitochondrial uniporter transports calcium from the cytosol into the mitochondrial matrix and is located on the inner mitochondrial membrane. SERCA is located on SR and ER membranes and couples ATP hydrolysis to the transport of calcium from the cytosol to the lumenal space. Removal of calcium can also be achieved through the use of calcium buffer proteins such as parvalbumin, calretinin and calbindin D-28 (reviews (Berridge et al., 2000; Berridge et al., 2003; Bootman et al., 2001; Carafoli, 2002; Cullen and Lockyer, 2002)).

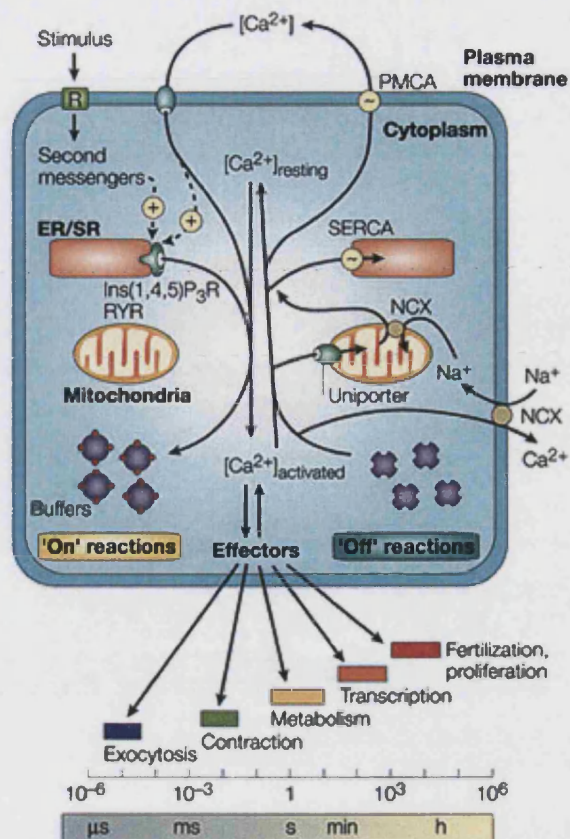


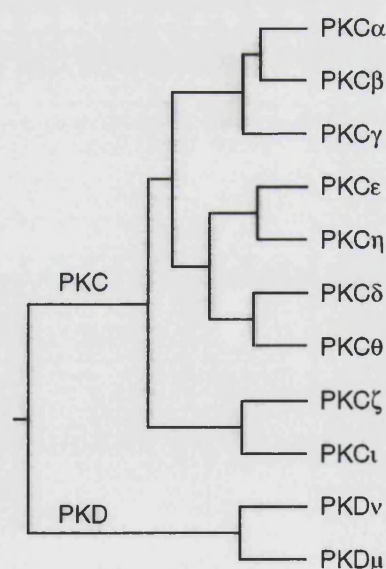
Figure 1.16. The ON and OFF mechanisms involved in calcium signalling. During the ON phase, stimuli induce both the entry of external calcium (red circles) and the formation of second messengers that release internal calcium stored within the ER/SR. During the OFF phase, calcium leaves the effectors and buffers and is removed from the cytoplasm by various exchangers and pumps. From (Berridge et al., 2003).

Rises in cytosolic calcium levels are decoded by various intracellular calcium binding proteins coupling the calcium flux to a biochemical and cellular response. EF hand proteins (see appendix 9) are calcium-binding proteins of which there are more than 600, with calmodulin being the most abundant and well known of these calcium sensors. Calmodulin binding to calcium leads to its conformational change and activation of the serine-threonine phosphatase calcineurin, allowing this protein to then activate the transcription factor nuclear factor of activated T cells (NFAT) and subsequent transcription of various genes, such as IL-2. Proteins containing C2 domains are also able to decipher calcium signals and these proteins include the classical protein kinase C isoforms (discussed in next section) (Carafoli, 2002). All chemokine receptors have been demonstrated to elicit a calcium flux upon ligation.

Protein Kinase C (PKC) Family

CLASSICAL					
					
ISOFORM	AMINO ACIDS	MW (calculated)	CHROMOSOMAL LOCATION	DAG or Ca ²⁺ ACTIVATED	EXPRESSION
PKC α	672	76,704	17q24	DAG/Ca ²⁺	Ubiquitous
PKC β_1	671	76,663	16p12	DAG/Ca ²⁺	Ubiquitous
PKC β_{II}	673	76,806	16p12	DAG/Ca ²⁺	Ubiquitous
PKC γ	697	78,268	19q13.4	DAG/Ca ²⁺	Brain/CNS
NOVEL					
					
PKC δ	674	77,458	3p21.1	DAG	Ubiquitous
PKC ϵ	737	83,492	2p21	DAG	Ubiquitous
PKC η	683	77,884	14q22-23	DAG	Ubiquitous
PKC θ	707	81,479	10p15	DAG	Ubiquitous
ATYPICAL					
					
PKC ζ	592	67,605	1p36.3	Neither	Ubiquitous
PKC λ	586	67,200	3q26	Neither	Ubiquitous
PKD					
					
PKC μ /PKD1	912	102,000	14q11	DAG	Kidney, lung
PKD2	878	97,000		DAG	Ubiquitous
PKC ν /PKD3	890	100,500	2p21	DAG	Ubiquitous

Figure 1.17. Schematic representation of PKC kinase family members and phylogenetic tree. Like many other signalling effectors, PKC is not a single entity but products of various mammalian PKC genes. The molecular weight (MW) is given as calculated MW and chromosomal location refers to human. The PKC family has traditionally been classified into 3 groups (classical, novel and atypical), however, it has recently become evident that PKC μ , originally classified by some as a member of the novel family, is the first member of a new group of kinases termed the protein kinase D (PKD) family (sometimes classified as a subgroup of the PKCs, but shares little structural homology with the others, as demonstrated by the phylogenetic tree – obtained from (Kofler et al., 2002)). *Abbreviations:* PS, pseudosubstrate; TM, transmembrane domain; PH, pleckstrin homology domain.



Protein kinase C (PKC) was first identified in 1977 in bovine cerebellum (Takai et al., 1977) and since then its involvement in many biological processes has been demonstrated, including development, memory, cell differentiation, migration, proliferation, carcinogenesis, and apoptosis (reviews (Baier, 2003; Dempsey et al., 2000; Liu and Heckman, 1998; Mochly-Rosen and Kauvar, 2000)). The PKCs are a large family of serine/threonine kinases consisting of at least 10 isoforms that can be split into three (or four) groups based upon their requirements of calcium and phorbol esters for activation (fig. 1.17). The classical PKC (cPKC) isoforms are calcium-dependent via their C2 domains and are activated by DAG, phosphatidylserine (PS) and phorbol esters (e.g. PMA) through their cysteine-rich C1 domains; novel PKC (nPKC) isoforms are calcium-independent but still regulated by DAG, PS and PMA; atypical PKC (aPKC) isoforms are calcium-independent and also don't require DAG for their activation (Mellor and Parker, 1998; Moscat and Diaz-Meco, 2000; Newton, 1995a; Newton, 1995b; Ron and Kazanietz, 1999; Way et al., 2000). The three classes of PKC mentioned above all possess a pseudosubstrate or auto-inhibitory domain that binds to the substrate-binding site in the catalytic (kinase) domain preventing its activation in the absence of activators (Orr and Newton, 1994). In recent years, distantly related PKC members have been discovered (PKC μ (human homologue of mouse PKD) (Johannes et al., 1994; Valverde et al., 1994), PKC ν (PKD3) (Hayashi et al., 1999) and PKD2 (Sturany et al., 2001)) and, like the nPKCs, are regulated by DAG (i.e. contain a C1 domain that is homologous to the C1 domains of the other PKC classes) and lack the C2 domain. However, similarities with the other classes end there: the catalytic domain is only 30-35% homologous to the catalytic domains of the other three classes of PKC isoforms; there is a PH domain in the regulatory region; the auto-inhibitory pseudosubstrate motif is absent; and the catalytic domain has distinctly different substrate specificities (for reviews of PKD see (Lint et al., 2002; Rykx et al., 2003; Van Lint et al., 2002)). This has recently led to the proposal by some researchers that this fourth group of PKCs is considered as a distinct group of new protein kinases, termed the PKD family. Another group of distantly related PKC enzymes are the PKC-related kinases (PRKs), also known as the protein kinase N (PKN) family. This group currently comprises 3 members (PKN α /PRK1, PKN β , PRK2/PKN γ) and are activated by Rho (not DAG or calcium) binding to the HR1 (homology region 1) domain (Mukai, 2003).

The activity of PKC is under the control of three distinct phosphorylation events (fig. 1.18). Upon ligand binding at the membrane, PKC becomes a substrate for kinases phosphorylating: 1). threonine (Thr497 for PKC α) in the activation loop (T-loop) by 3-phosphoinositide-dependent protein kinase 1 (PDK1) in many instances (Le Good et al., 1998; Sonnenburg et al., 2001), rendering the kinase catalytically competent to autophosphorylation; 2). kinase-mediated/autophosphorylation of serine (S657 for PKC α) in the hydrophobic C-terminal site (Keranen et al., 1995); 3). autophosphorylation of threonine (Thr638 for PKC α) in the C-terminus (the 'turn motif') results in the kinase domain taking up a closed conformation state that confers stability and phosphatase resistance (Parekh et al., 2000). The α PKCs lack phosphorylation at the hydrophobic site due to the presence of a glutamic acid residue in place of the serine.

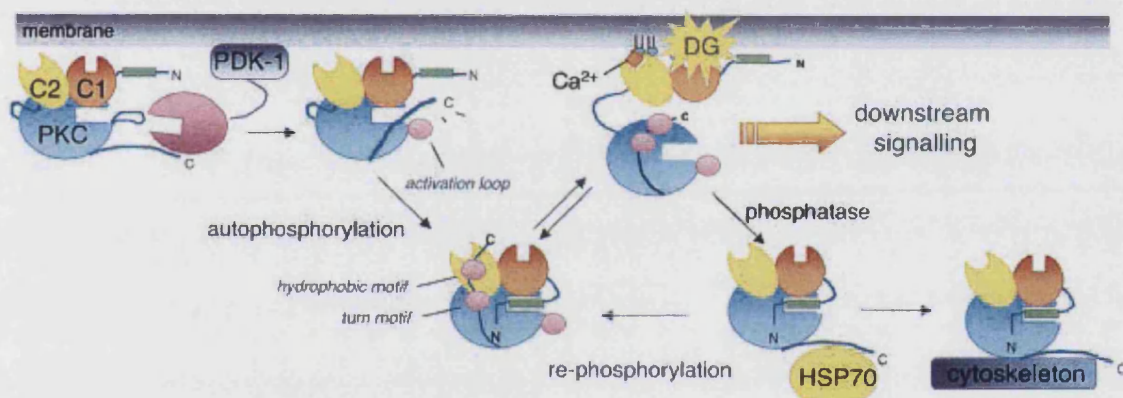


Figure 1.18. Regulation of PKC activity. See text for details on the phosphorylation events. The molecular chaperone Hsp70 binds the dephosphorylated turn motif and stabilizes PKC, allowing it to become rephosphorylated and to re-enter the pool of signalling-competent PKC. In the absence of Hsp70 binding, or as a result of chronic activation, dephosphorylated PKC accumulates in a detergent-insoluble cell fraction, where it is eventually degraded (Newton, 2003).

Inactive PKC isoforms are able to bind a number of scaffolding proteins including A kinase-anchoring proteins (AKAPs) that may serve to localise PKC enzymes to specific sites within the cell and thus helping to regulate cellular activities (Dell'Acqua et al., 1998; Faux et al., 1999; Faux and Scott, 1997; Klauck et al., 1996). Activated PKC enzymes bind proteins and substrates including STICKs (Substrates That Interact with C-Kinase), RACKs

(Receptors for Activated C-Kinase – not a substrate), MARCKS (myristoylated alanine-rich C-kinase substrate), actin, PLD (phospholipase D), and calmodulin (Jaken and Parker, 2000). PKC-mediated signalling pathways, as well as cross-talking effects (i.e. not direct), include the MAPK (mitogen-activated protein kinase) pathways, PKB (protein kinase B), PKD, IKK (IkB-Kinase), and NFAT (Baier, 2003). Chemokine receptors have been implicated in eliciting the activation of various PKC isoforms upon ligation (Carnevale and Cathcart, 2003; Ni et al., 2003; Pollok-Kopp et al., 2003).

Diacylglycerol (DAG), More Than Just PKC Activation

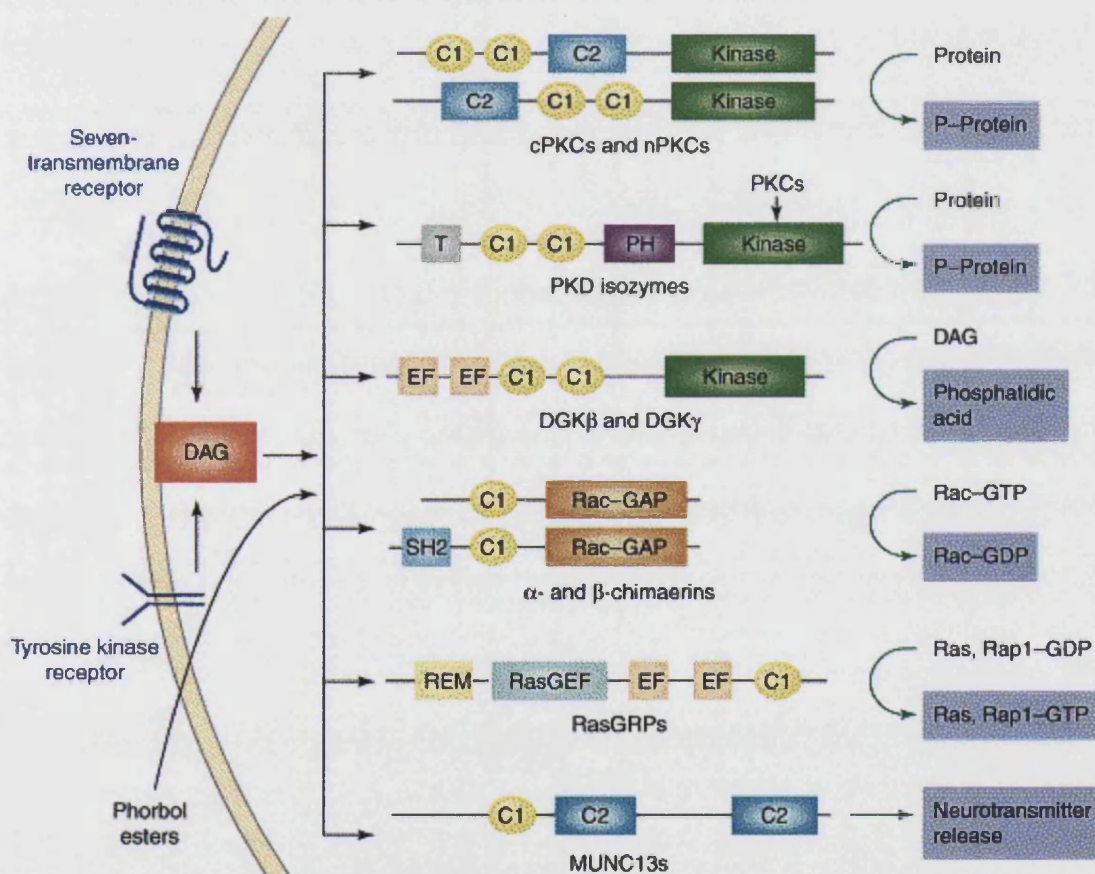


Figure 1.19. DAG stimulation of PKC-dependent and independent signalling pathways. The generation of DAG, through stimulation of membrane receptors, leads to the activation of various proteins that possess DAG- and phorbol ester-responsive C1 domains (Yang and Kazanietz, 2003).

T lymphocytes contain up to 8 of the PKC isozymes making it difficult to elucidate the function of specific PKC isoforms, especially with the lack of isoform selective inhibitors. In addition to the use of pharmacological inhibitors, functions have been attributed to PKC through the use of prolonged phorbol ester treatment. However, it has become clear that DAG activates a number of unrelated targets and more than just PKC, with this concept largely being ignored in the past. Figure 1.19 highlights some of these DAG targets (for much more in depth reviews see (Kazanietz, 2002; Ron and Kazanietz, 1999; Yang and Kazanietz, 2003).

Phosphatidylinositol 3-Kinase (PI3K)

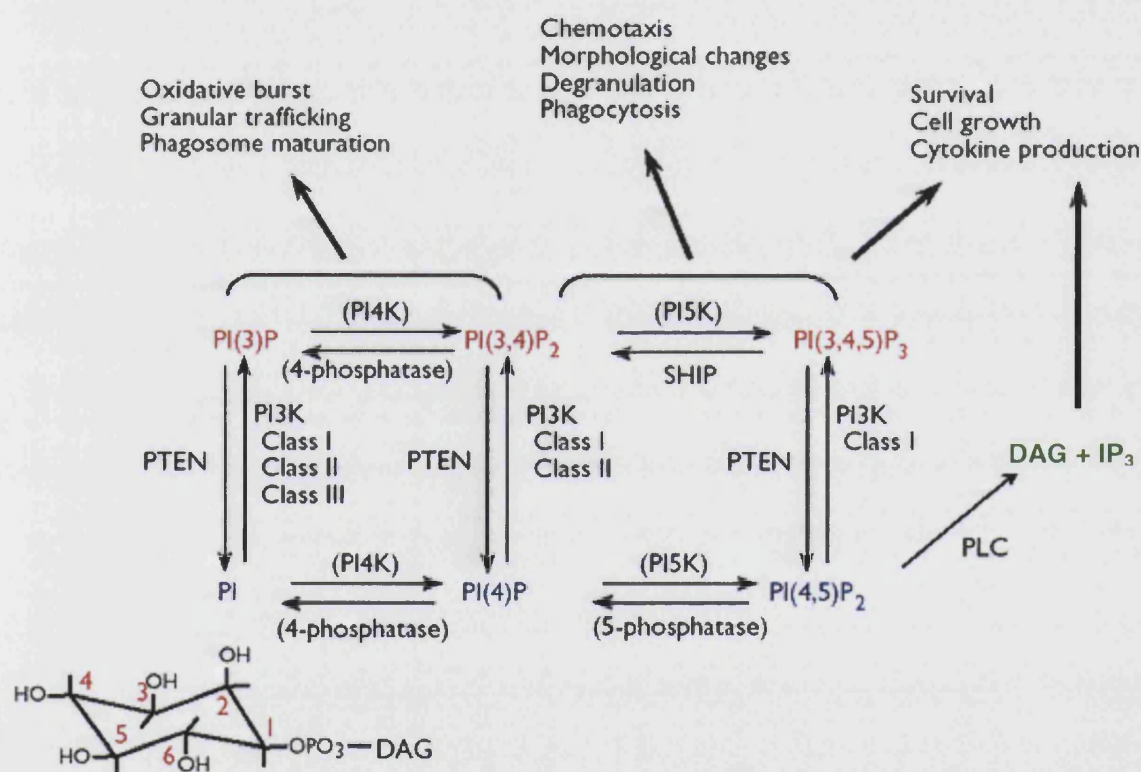


Figure 1.20. Metabolism and immune system functions of phosphoinositides. Phosphorylation of the 4- and 5-position of the inositol ring is catalysed by distinct phosphoinositide kinases. Phosphoinositide 3-kinase (PI3K) products are shown in red. There are also several 3-, 4- and 5-phosphatases (Koyasu, 2003). *Abbreviations (not defined in text):* SHIP, 5' phosphatase Src Homology 2 domain-containing inositol polyphosphate; PTEN, 3' phosphatase and tensin homologue deleted on chromosome 10.

Over the last few years the extent and variety of the role the phosphoinositides (PIs) play in cellular signalling has emerged. Besides PI(4,5)P₂, mentioned above, the precursor of the intracellular second messengers DAG and Ins(1,4,5)P₃, there have been many other metabolic pathways identified that lead to the generation of seven different PIs (for a general review of PIs in cell signalling see the review by (Payraastre et al., 2001)). A few of these appear to play major roles in key signalling pathways, with the D3 PIs playing, perhaps, the most prominent duties. The phosphatidylinositol 3-kinases (PI3Ks) are responsible for the generation of these D3 PIs (fig. 1.20). The multiple isoforms of PI3Ks (fig. 1.21) are able to phosphorylate the 3'-OH position of the myo-inositol ring of inositol phospholipids to produce phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂), and phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃) (fig. 1.12, in 'PLC' section – incidentally, the D3 PIs are not substrates for known PLC isoforms). These 3'-phosphoinositides have been demonstrated, through the use of pharmacological tools and gene targeting techniques, to govern many cellular events, including cell growth and survival, cytoskeletal remodelling, migration, differentiation, and adhesion (fig. 1.20) (Katso et al., 2001; Koyasu, 2003; Okkenhaug and Vanhaesebroeck, 2003a; Vanhaesebroeck et al., 2001). Furthermore, the importance of PI3K is evident from the various knockout studies that have been performed into each of the class I PI3K isoforms. The p110 α and p110 β knockout mice display embryonic lethality and the class IA p85 α adaptor knockout is perinatally lethal. The p110 δ and PI3K γ knockouts are not lethal but display defective immune functions consistent with their restricted expression in leukocytes (Okkenhaug and Vanhaesebroeck, 2003b; Sasaki et al., 2002).

The PI3K family can be subdivided into 3 classes based on structural similarities and substrate specificity – class I, class II, and class III (fig.1.21). All PI3K catalytic subunits share a homologous region that consists of the catalytic domain (homology region 1 (HR1)) linked to a PIK (phosphatidylinositol kinase) domain (HR2) and a C2 domain (HR3). The PIK (helical) domain is found in lipid kinases but not protein kinases, and acts as the structural spine around which the other domains are anchored (Djordjevic and Driscoll, 2002). The C2 domains of PI3K likely bind phospholipid in a calcium-independent manner (contrary to the C2 domains found in other kinases, such as cPKC isoforms). Although the C2 domains of class I PI3Ks are similar in structure to the C2 domains of PKC δ , PLC δ and

cPLA2, they lack the necessary residues to coordinate calcium binding and therefore may be involved in calcium-independent events. The catalytic domain is divided structurally into two lobes. The N-terminal lobe contains the nucleotide-binding loop (P-loop) with a conserved lysine residue within the ATP binding pocket and is critical for the correct alignment of ATP with the active site. The active site is found in the activation loop of the second lobe (the C-terminal lobe). This region, in addition to contributing to ATP binding, contains the activation and catalytic loops. The activation loop determines substrate specificity and the catalytic loop is responsible for deprotonating the substrate, generating the nucleophile that attacks the γ -phosphate of ATP (Vanhaesebroeck et al., 2001).

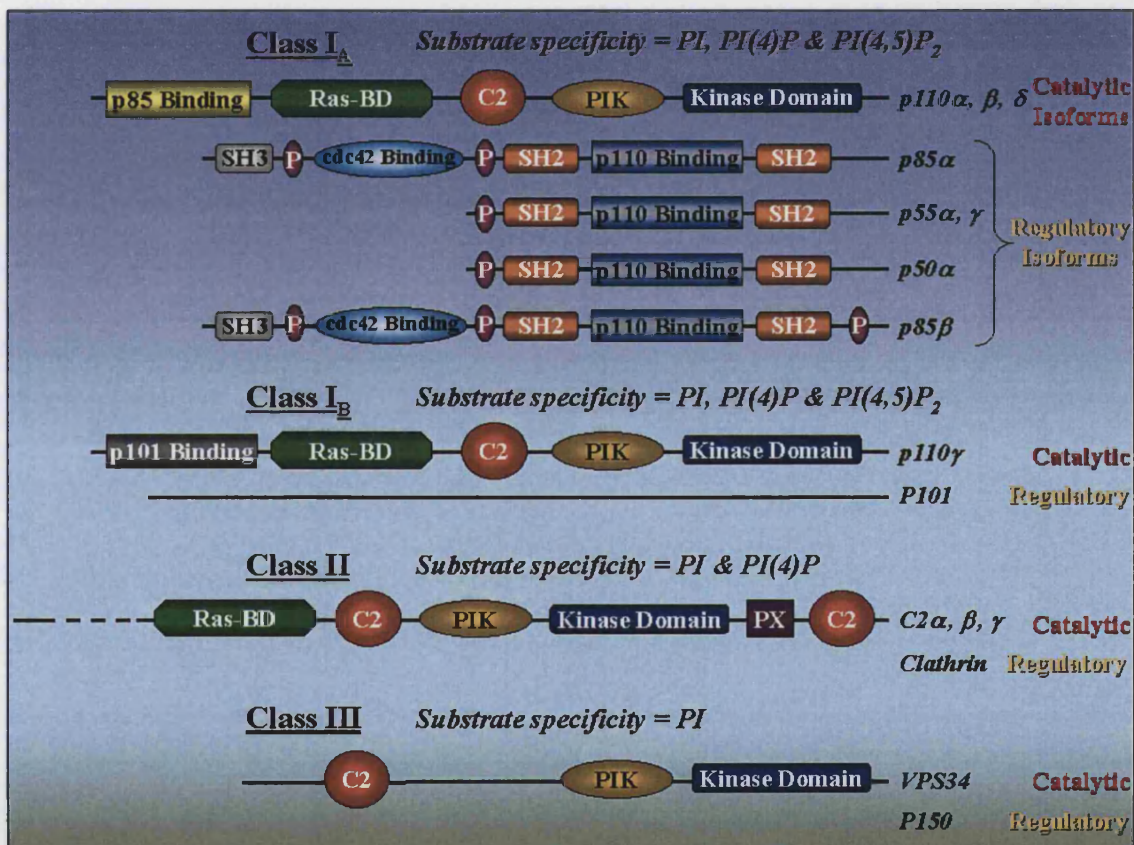


Figure 1.21. The classification of PI3K family members and schematic structures. The PI3K family consists of 3 classes of isoforms, with each class demonstrating varying substrate specificities. A catalytic and a regulatory isoform associate to generate a biologically active complex. *Abbreviations:* Ras-BD, Ras-binding domain; PIK, phosphatidylinositol kinase domain (also known as the 'helical domain'); SH2, Src homology 2 domain; SH3, Src homology 3 domain; PX, Phox homology domain; P, proline-rich motif; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.

Class I PI3Ks

The class I PI3Ks are further divided into 2 groups based on structural and functional differences - class I_A and class I_B. The class I_A PI3Ks are heterodimers comprising a regulatory subunit (p85 α , p55 α , p50 α (all 3 derived from a single gene by alternative splicing (Fruman et al., 1996)), p85 β or p55 γ (each encoded by distinct genes)) and a catalytic subunit (p110 α , p110 β , or p110 δ). The p85 α subunit is the most abundantly expressed of the regulatory adaptors. The p110 α and β catalytic subunits are ubiquitously expressed whilst the p110 δ subunit is restricted to leukocytes. Each of the catalytic subunits can associate with all of the regulatory subunits and the catalytic subunits are constitutively associated with a regulatory subunit (maintains stability of p110). The class I_A regulatory subunits contain two SH2 domains that bind specific phosphorylated tyrosine residues in proteins located at the plasma membrane, thus bringing the catalytic subunit to within proximity of its substrate. This leads to the activation of the PI3K catalytic subunit, which is normally inhibited in the p85/p110 complex (Koyasu, 2003; Yu et al., 1998b). The class I_A PI3Ks are activated by tyrosine kinase receptors (cytokine, antigen, etc) and to a lesser extent GPCRs (G-protein coupled receptors).

The class I_B PI3K (PI3K γ) is only found in mammals and, like p110 δ , expression is restricted to leukocytes. PI3K γ is similar in structure and function to the class I_A PI3K but there is only one catalytic subunit (p110 γ) and one regulatory subunit (p101). PI3K γ is the only PI3K isoform that is specifically activated by the GPCR-associated small G proteins G $\beta\gamma$ (Brock et al., 2003; Krugmann et al., 1999; Stephens et al., 1997).

The class I PI3Ks are able to utilise PI, PI(4)P and PI(4,5)P₂ as substrates *in vitro*, however, PI(4,5)P₂ is the main substrate *in vivo* (Katso et al., 2001).

Class II PI3Ks

This class currently contains three members: PI3K-C2 α , PI3K-C2 β , and PI3K-C2 γ . C2 α and C2 β are ubiquitously expressed, whereas C2 γ expression is restricted to the liver (Koyasu, 2003). This class differ significantly from the class I PI3Ks in both their mode of

regulation and substrate preference (PI and PI(4)P *in vitro*, but preferred *in vivo* substrate is unknown). These enzymes are much larger (~ 170 kDa) than class I PI3Ks and have two distinct domains in the C-terminal: a Phox (PX) domain and another C2 domain (fig. 1.21). The PX domain binds PI(3)P and PI(3,4)P₂ whereas the C2 domain function is unclear. The class II PI3Ks have been demonstrated to be activated downstream of growth factor receptors, cytokine/chemokine receptors, receptor tyrosine kinases and integrin receptors (Arcaro et al., 2000; Brown et al., 1999; Turner et al., 1998; Urso et al., 1999). The class II PI3Ks have been relatively under-investigated compared to their class I PI3K counterparts and thus, moderately little is known about them.

Class III PI3Ks

Consisting of a single member, Vps34p (the homologue of the *Saccharomyces cerevisiae* yeast vesicular-protein-sorting protein), it exclusively generates PI(3)P from PI and is broadly expressed in complex with a Ser/Thr protein kinase termed p150 (Vps15p in yeast). Its thought that, along with class II PI3Ks, it plays a role in vesicular trafficking (Simonsen et al., 2001; Wurmser et al., 1999). However, very little is known about the activation of the class III PI3Ks *in vivo*.

Chemokine-Mediated PI3K Activation

Various chemokines have been demonstrated to elicit a PI3K response in different cell systems (Knall et al., 1997; Sotsios et al., 1999; Turner et al., 1998). PI3K γ is the only G $\beta\gamma$ -dependent PI3K and it is believed that it has a central role to play in chemokine-mediated PI(3,4,5)P₃ accumulation and migration (fig. 1.22) (Al Aoukaty et al., 1999; Hirsch et al., 2000; Wu et al., 2000). The most widely studied lipid kinase is the p85/p110 PI3K, and its role in chemokine-stimulated PI(3,4,5)P₃ accumulation is somewhat controversial. There is plenty of evidence to suggest that p85/p110 is activated (Kurosu et al., 1997; Turner et al., 1998; Vicente-Manzanares et al., 1999) but there are doubts about how much it contributes to PI(3,4,5)P₃ levels. An excellent example of this is the CCL2-stimulated PI(3,4,5)P₃ accumulation is inhibited by PTX (pertussis toxin) but yet activation of p85/p110 is PTX-independent (Turner et al., 1998). It is possible that the G α_i subunits bind and activate Src

(and other tyrosine kinases) which can then lead to the activation of the p85/p110 heterodimer and the subsequent activation of distinct signalling pathways. Indeed, in $PI3K\gamma^{-/-}$ mice there was incomplete inhibition of cell migration in response to various chemokines (Sasaki et al., 2000). It is therefore likely that various PI3Ks are involved in cell motility. Finally, the class II PI3K, PI3K-C2 α , has been shown to lie downstream of the MCP-1/CCL2 chemokine receptor (CCR2) but the functional relevance of this class is unknown (Turner et al., 1998). For reviews see (Curnock et al., 2002; Rickert et al., 2000; Sotsios and Ward, 2000; Stephens et al., 2002).

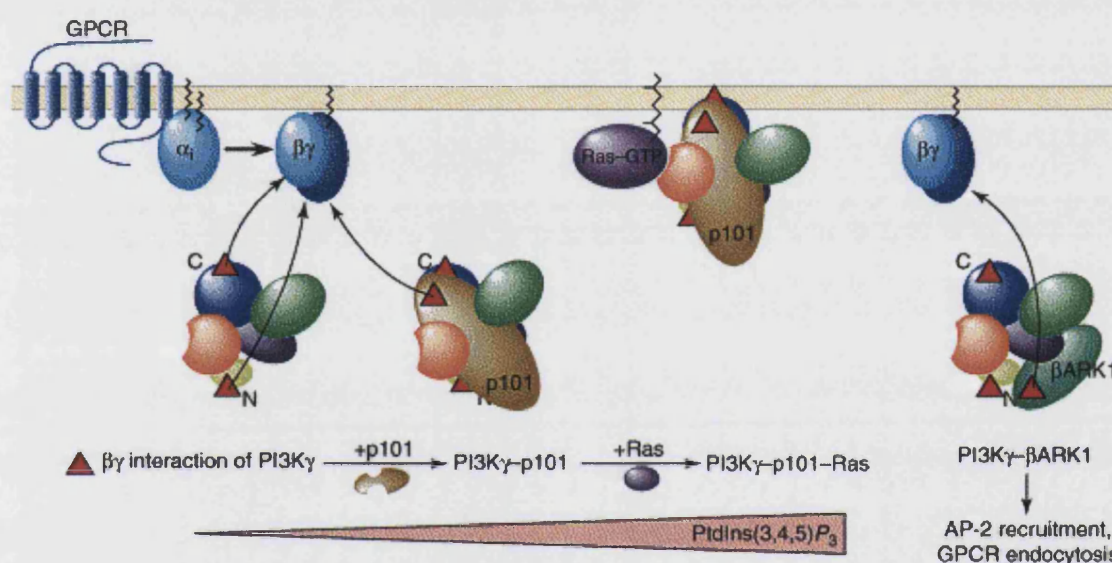


Figure 1.22. Activation of class I_B PI3K γ by G-protein-coupled receptors (GPCRs). Binding of a ligand to GPCRs initiates the dissociation of heterotrimeric G proteins, which liberates $\beta\gamma$ -subunits that interact with the N- and C-terminal regions of PI3K γ . This triggers translocation and allosteric activation of PI3K γ . PI3K γ is associated tightly with p101 through interactions in the adaptor-binding domain and the C-terminal lobe of the catalytic domain. The presence of p101 sensitizes PI3K γ to activation by $\beta\gamma$ -subunits. Interaction with GTP-bound Ras is proposed to induce conformational changes and membrane reorientation of the lipid kinase in relation to its substrate, PI(4,5)P₂, thus further increasing its activity. Although PI3K γ - $\beta\gamma$ -p101-Ras interaction leads to massive production of PI(3,4,5)P₃ (e.g. in neutrophils), the sequence of actions and the quantitative contribution of PI3K γ -interacting proteins in physiological processes need further elucidation. The interaction of β -adrenoceptor kinase 1 (β ARK1) with the PI3K helical domain (also called the PIK domain) of PI3K γ is instrumental for the localized production of PI(3,4,5)P₃ that is required for the recruitment of the clathrin adaptor AP-2 (possibly via the μ 2 subunit of the AP2 complex) and endocytosis of β 2-adrenoceptors. Interestingly, inhibition of the PI3K γ - β ARK1 interaction interferes with endocytosis of β 2-adrenoceptors, but not with PI3K γ -mediated activation of protein kinase B (Naga Prasad et al., 2002; Wymann et al., 2003b).

Phosphoinositide-Binding

PI is the most abundant phosphoinositide in mammalian cells (10% of total glycerophospholipids) with phosphorylations at the D3, D4, and D5 positions by specific kinases leading to the generation of 7 different polyPI products. PI(4)P and PI(4,5)P₂ each represent about 10% of total PIs (1% total glycerophospholipids) and, along with PI, levels are kept at a steady state in cell membranes. The PI3K products, PI(3,4)P₂ and PI(3,4,5)P₃, are rapidly and transiently produced in response to agonist and their levels never exceed 10% of PI(4,5)P₂ levels (0.1% total glycerophospholipids). PI(3)P is constitutively present in small quantities and the levels are relatively stable in mammalian cells, unlike PI(5)P and PI(3,5)P₂, that although present in relatively small amounts, their levels do alter upon cell activation (Pendaries et al., 2003). A plethora of proteins display an ability to interact with these phospholipids via various PI-binding motifs. These interactions vary from high to low affinity and the different domains have a preference for specific PIs (table 1.7). Hence, the activity of PI3K is able to regulate cellular processes via their ability to generate a variety of D3-PIs that act as site-specific membrane signals for a range of effector proteins. Following is a brief description of some of the more relevant domains found to date.

Epsin NH₂-Terminal Homology (ENTH) Domain

First found in the endocytic protein epsin 1, it is a region of approximately 140 amino acids usually located in the N-terminus with binding selectivity for PI(4,5)P₂. It is found predominantly in endocytic and cytoskeletal organisational proteins such as AP180 (adaptor protein 180) and CALM (clathrin assembly lymphoid myeloid leukaemia protein). Compared with the other PI-binding domains mentioned, very little detail is known about the role of this domain in cellular processes (Itoh and Takenawa, 2002; Xu et al., 2001).

Band 4.1, Ezrin, Radixin, Moesin (FERM) Domains

There are more than 40 FERM domain-containing proteins with the majority serving as regulated cross-linkers connecting actin filaments to membranes via the specific binding of PI(4,5)P₂ lipids. Additionally, in many cases they also possess a spectrin/actin-binding domain (SABD) to facilitate this role. As a consequence, FERM proteins have been implicated in cytoskeletal processes, such as reorganisation (DiNitto et al., 2003).

Fab1p, YOTB, Vac1p and EEA1 (FYVE) Domain

The FYVE domain is a compact double Zn^{2+} finger module and was first identified in the four proteins it derives its name from (see heading) and is a ~60 – 80 amino acid domain that consists of 8 conserved cysteine residues that are characteristic of the zinc-finger. There is a cluster of basic residues that surround the third and fourth cysteines [R(R/K)HHCR] and this positively charged sequence, along with Zn^{2+} (2 atoms), is essential for the folding of the zinc finger and the electrostatic interaction with the negatively charged head group of PI(3)P (its specific PI target). The FYVE domain is thought to play a significant role in membrane/vesicular trafficking due to the specific localisation of PI(3)P to the membranes of early endosomes and to the internal vesicles of multivesicular bodies (Gillooly et al., 2003; Itoh and Takenawa, 2002; Vanhaesebroeck et al., 2001; Xu et al., 2001).

Pleckstrin Homology (PH) Domains

PH domains have been found in a wide variety of proteins (> 250). It was first identified as a 100 – 200 amino acid sequence homology domain found originally in the PKC substrate pleckstrin (in platelets). However, there is limited sequence similarity between the PH domains of different molecules (typically 10 – 20%) and are defined instead by a conserved tertiary structure (Xu et al., 2001). The PH domains are able to bind most of the doubly/triply phosphorylated PIs (mostly PI(3,4)P₂, PI(4,5)P₂ and PI(3,4,5)P₃) but do display, in general, greater selectivity for the D3 PIs (table 1.7). The PH domains are classified into 3 groups based on their selectivity for PIs (Group 1 = bind a specific PI; Group 2 = demonstrate a low specificity and/or affinity; Group 3 = bind non-specifically to PIs) (Cullen et al., 2001; Maffucci and Falasca, 2001).

Phox (PX) Domains

The PX domain averages 100 – 120 amino acids in size and was first identified in two of the cytosolic components of NADPH oxidase, p40^{phox} and p47^{phox}. It has since been identified in a number of proteins (~ 65) that are implicated in diverse biological processes. Interestingly, the PX domain contains a polyproline motif (PxxP) in the middle that is characteristic of SH3 domain-binding motifs, and there is evidence that PX may be an

interacting partner of SH3 domains. The PX domain is generally considered to have binding selectivity for the D3 PIs (Ellson et al., 2002; Itoh and Takenawa, 2002; Wishart et al., 2001; Xu et al., 2001).

Table 1.7. Phosphoinositide-binding domains.

DOMAIN	PROTEIN	SPECIFICITY
PH	PLC δ 1	PI(4,5)P ₂
	Btk	PI(3,4,5)P ₃
	GRP-1	PI(3,4,5)P ₃
	PLC β 1	PI(3)P, PI(4,5)P ₂ , PI(3,4,5)P ₃
	β -ARK	PI(3,4,5)P ₃ , PI(4,5)P ₂
	Spectrin	PI(3,4,5)P ₃ , PI(4,5)P ₂
	mSos	PI(3,4,5)P ₃ , PI(4,5)P ₂
	Akt/PKB	PI(3,4)P ₂ , PI(3,4,5)P ₃
	Pleckstrin	Non-specific
	DAG δ	Non-specific
FYVE	EEA1	PI(3)P
	Hrs	PI(3)P
	Vac1p	PI(3)P
	Vps27p	PI(3)P
	Pib1p	PI(3)P
	Fab1p	PI(3)P
PX	SNX3	PI(3)P
	SNX7	PI(3)P
	p40 ^{phox}	PI(3)P
	p47 ^{phox}	PI(3,4)P ₂ , PI(3)P, PI(3,5)P ₂ , PI(3,4,5)P ₃
	Vam 7p	PI(3)P
	Bem 1p	PI(3)P, PI(5)P
	PI3K-C2 α	PI(4,5)P ₂
	CISK	PI(3,5)P ₂ , PI(3,4,5)P ₃
ENTH	Epsin 1	PI(4,5)P ₂
	Epsin 2b	PI(4,5)P ₂
	AP180	PI(4,5)P ₂
	Hip1	PI(4,5)P ₂
FERM	Ezrin	PI(4,5)P ₂
	Radixin	PI(4,5)P ₂

This table is derived and modified from those published by (Itoh and Takenawa, 2002; Maffucci and Falasca, 2001). PH domains highlighted in **red** are classified as Group 1 PH domains, **blue** are Group 2 PH domains, and **green** are Group 3 PH domains. The PX domains are divided into 3 groups based on the PX domain representing more than half of the polypeptide (**red**), have longer flanking sequences but contain no other motifs (**blue**), and proteins that contain other characterised domains such as SH3 and PH domains (**green**).

Downstream Effectors of PI3K: PKB and company

The PI3K-mediated signalling pathways have been one of the most intensely studied areas of signal transduction over the last decade or so. Thus, vast arrays of studies have been carried out into multitude of cellular processes searching for roles of the PI3K isoforms. Consequently, PI3K has been found to play a central role in control of metabolism, cell growth, proliferation, survival, migration, and membrane transport and secretion. As would be expected from the variety of cellular processes PI3K has been implicated in, there have been discovered a vast number of proteins that lie downstream of the products of PI3K and, therefore, a variety of signalling cascades (fig. 1.23). Discussed below are a few of the key proteins involved in these signalling pathways and that will be mentioned during this thesis (for more in depth reviews read (Cantley, 2002; Cantrell, 2001; Seminario and Wange, 2002; Vanhaesebroeck and Alessi, 2000; Ward and Cantrell, 2001)).

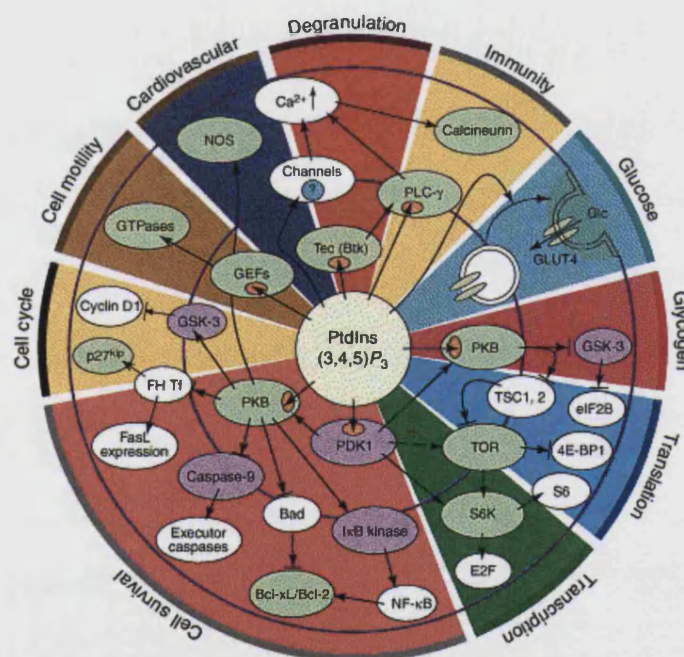


Figure 1.23. A PI3K pathway dartboard. PI(3,4,5)P₃ produced by class I PI3Ks recruits proteins that contain PH domains (among other D3 PI-binding domains), including PKB, phosphoinositide-dependent kinase 1 (PDK1), guanine nucleotide exchange factors (GEFs), protein tyrosine kinases of the Tec family (e.g. Bruton's tyrosine kinase (Btk)), phospholipase C γ (PLC γ). PI3K exerts control on most cellular processes and, although this control is central, the signalling pathways depicted intersect with parallel, non-PI3K-dependent mechanisms (not shown) (Wymann et al., 2003b). *Abbreviations:* 4E-BP1, eukaryotic initiation factor 4E binding protein 1; Glc, glucose; GLUT4, glucose transporter 4; I κ B, inhibitor of NF κ B; NF κ B, nuclear factor κ B; TSC1, hamartin; TSC2, tuberlin.

3'-Phosphoinositide-Dependent Kinase (PDK) 1 & 2

The AGC (named after its original constituent members, the protein kinases A, G, and C) superfamily of serine/threonine kinases was recognised as a group of enzymes that were a key mediator of the PI3K signal, but the majority of its members required phosphorylation for activation. This led to the search for a potential upstream kinase that was linked to the PI3K pathway and this search culminated in the discovery of 3'-phosphoinositide-dependent kinase-1 (PDK-1) in 1997, initially purified from rabbit skeletal muscle and rat brain and cloned later that year (Alessi et al., 1997a; Alessi et al., 1997b; Stokoe et al., 1997).

PDK-1 is a 63 kDa serine/threonine kinase ubiquitously expressed in human tissues and is responsible for the phosphorylation of PKB on Thr 308. The ability of PDK-1 to carry out this activity relies on the presence of PI(3,4)P₂ and PI(3,4,5)P₃, resulting in the kinase obtaining its current name (Vanhaesebroeck and Alessi, 2000). PDK-1 possesses a N-terminal kinase domain that shares homology with the catalytic domains found in the cAMP-dependent protein kinase A (PKA) and PKC subfamilies, and a C-terminal PH domain that is responsible for binding to the PI(3,4)P₂ and PI(3,4,5)P₃ lipids (Brazil and Hemmings, 2001). The activity of PDK-1 to phosphorylate PKB relies upon the conformation of PKB. Specifically, the PH domain of PKB is required to bind PI(3,4)P₂ or PI(3,4,5)P₃ lipids in order to remove the autoinhibition of the active site of PKB, allowing PDK-1 to access Thr 308 on the activation loop and phosphorylate the residue (fig. 1.24) (Toker and Newton, 2000b). However, PDK-1 is only able to phosphorylate Thr 308 on PKB and is unable to phosphorylate Ser 473 in the hydrophobic motif of PKB, which is additionally required for full PKB activity. PDK-1 appears to exist in an active, phosphorylated configuration predominantly localised in the cytosol under basal conditions and possibly translocates to the membrane in response to increased D3 PI production (Vanhaesebroeck and Alessi, 2000).

Over the years, the kinase responsible for this phosphorylation of Ser 473 has remained elusive, and it was widely accepted early on that a separate kinase was responsible for this phosphorylation and was tentatively termed PDK-2. This has led to at least three models for Ser 473 phosphorylation in this time: 1). PDK-2 is a modified form of PDK-1, with the interaction of PDK-1 with a fragment of the C-terminal of PRK2 (protein kinase C-related

kinase 2 – mentioned earlier), termed PDK-1-interacting fragment (PIF), converts PDK-1 into an enzyme that is able to phosphorylate both Thr 308 and Ser 473 of PKB α *in vitro* (Balendran et al., 1999); 2). PKB autophosphorylation of Ser 473, similar to that observed with the regulation of the hydrophobic site in the cPKC isoforms – phosphorylation of Thr 308 by PDK-1 results in partial activation of PKB allowing it to autophosphorylate itself (Toker and Newton, 2000a); 3). PDK-2 is a distinct kinase – cells lacking PDK-1 demonstrate no phosphorylation of Thr 308 in PKB upon stimulation of cells with growth factor, but phosphorylation of Ser 473 still occurs (Williams et al., 2000). A recent study has provided a strong argument for PDK-2 being a cytoskeleton-associated kinase distinct from PDK1 (Hresko et al., 2003).

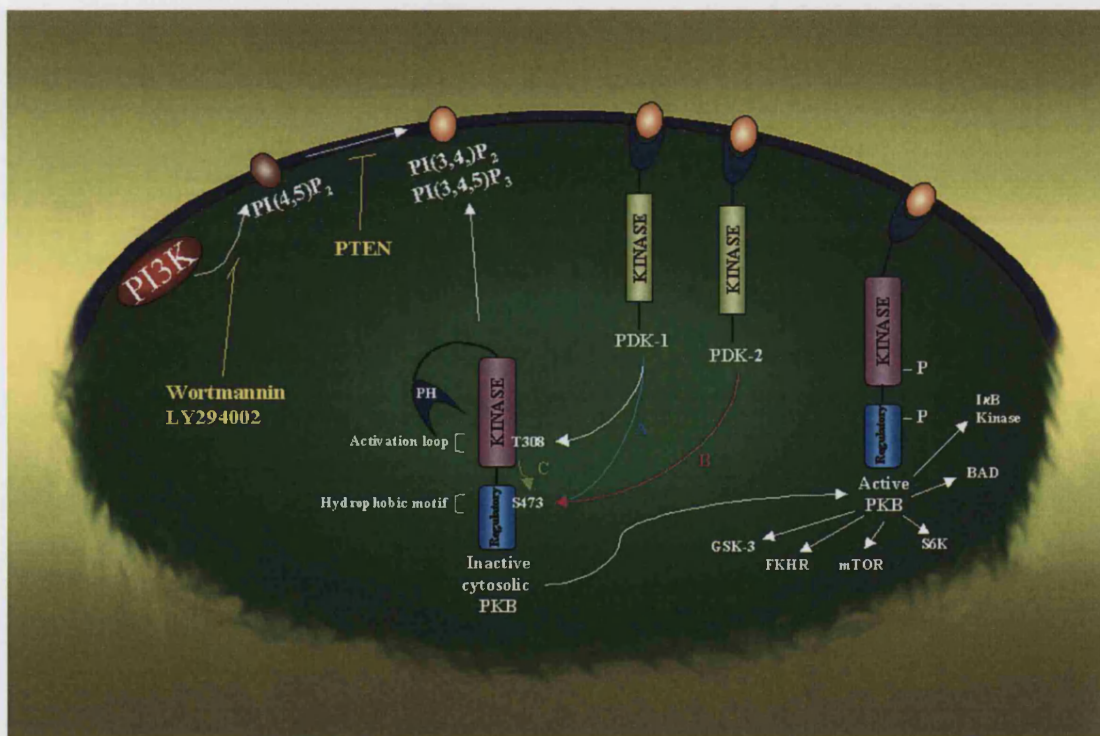


Figure 1.24. Role of PDK-1/2 in the activation of PKB. In unstimulated cells, PKB is not phosphorylated at either Thr 308 or Ser 473 and resides predominantly in the cytosol. Stimulation of PI3K leads to the accumulation of D3 PIs, thereby recruiting PKB to the membrane via its PH domain, where it is phosphorylated on Thr 308 by PDK-1 (which is constitutively recruited to the membrane via its PH domain) and on Ser 473 by an unknown mechanism, but may involve: (A) phosphorylation by a modified form of PDK-1; (B) phosphorylation by a distinct kinase termed PDK-2; or (C) autophosphorylation of Ser 473 by the kinase domain. Activated PKB translocates to various parts of the cell where it activates a number of downstream effector molecules.

There is evidence that PDK-1 does not solely act as an activator of PKB, and that it may be involved in the activation of other AGC kinases such as PKC, p70S6K, SGK, PAK, RSK and PRK, with the activation of these molecules likely to be similar to that of PKB (Toker and Newton, 2000b; Vanhaesebroeck and Alessi, 2000). Indeed, PDK-1 is essential for T cell development and along with the evidence that it regulates multiple pathways has led to PDK-1 being increasingly referred to as a 'master kinase' (Hinton et al., 2004).

Protein Kinase B (PKB)

A significant breakthrough in understanding how membrane-bound lipids convey signals to the cytosol was made with the identification of PH domains. Protein kinase B (PKB) contains a PH domain that specifically binds PI3K lipid products (preferentially PI(3,4)P₂ and PI(3,4,5)P₃), and thereby establishing a firm link between PI3K and PKB signalling (James et al., 1996; Stephens et al., 1998). PKB is a family of 57 kDa serine/threonine kinases with a high homology to the protein kinase A and C families, and was first cloned in 1991 (Bellacosa et al., 1991; Coffey and Woodgett, 1991; Jones et al., 1991b; Jones et al., 1991a). There are three PKB isoforms, PKB α /Akt1, PKB β /Akt2 and PKB γ /Akt3, with each consisting of an N-terminal PH domain, a kinase domain, and a C-terminal regulatory tail. PKB α needs to be phosphorylated at both Thr 308 (kinase domain or T-loop) and Ser 473 (C-terminal regulatory/hydrophobic domain) for full activation (with PKB β at Thr 309/Ser 474 and PKB γ at Thr 305/Ser 472 (a splice variant of PKB γ has been reported (PKB γ 1) and lacks the Ser 472 site (as only 465 aa long) and may limit its potential) (Nicholson and Anderson, 2002)), and are ubiquitously expressed (PKB γ is in particularly large amounts in the brain, lung and kidney). PKB isoforms are phosphorylated at the T-loop by PDK1, with a number of possible mechanisms regulating the phosphorylation of Ser 473 (see 'PDK-1' section and fig. 1.24). PKB activation is rapid and the site of action of the activated kinase differs between cell types, with activation solely dependent upon PI3K activity, as demonstrated by complete inhibition of PKB activity with PI3K inhibitors (Alessi et al., 1996). Epithelial cells display active PKB at the membrane, whereas in lymphocytes, PKB dissociates from the membrane to the cytosol and enters the nucleus (the mechanism for this is unknown) (Cantrell, 2002). During this time, PKB phosphorylates a plethora of proteins that include glycogen synthase kinase-3, BAD,

FKHR, p70S6K, eNOS and Raf (for reviews see (Brazil and Hemmings, 2001; Lawlor and Alessi, 2001; Nicholson and Anderson, 2002; Scheid and Woodgett, 2001)). To date, all PKB substrates (fig. 1.23 & 1.24) contain the motif: Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa are preferably small residues other than glycine, and Hyd is a bulky hydrophobic residue (leucine or phenylalanine). PKB has been shown to have a central role to play in apoptosis, proliferation (cancer), insulin signalling (diabetes), and migration (fig. 1.23). A number of chemokines have been observed to activate PKB, including SDF-1/CXCL12, RANTES/CCL5, IL-8/CXCL8 and MIP-1 α /CCL3 ((Lentzsch et al., 2003; Sotsios et al., 1999; Tilton et al., 1997)) and this is prevented in PI3K $\gamma^{-/-}$ -deficient neutrophils with regards to IL-8/CXCL8 (Hirsch et al., 2000).

Glycogen Synthase Kinase-3 (GSK-3)

A serine/threonine kinase, glycogen synthase kinase-3 (GSK-3) was first isolated and purified as an enzyme capable of phosphorylating and, in so doing, inhibiting the enzyme glycogen synthase. GSK-3 was first isolated in 1980 and in the mid-90s it was discovered that PKB was responsible for the phosphorylation and inhibition of GSK-3 (Cross et al., 1995; Embi et al., 1980). Currently, there are two mammalian GSK-3 isoforms identified, GSK-3 α (phosphorylated at Ser 21/Tyr 279) and GSK-3 β (phosphorylated at Ser 9/Tyr 216), with molecular weights of 51 and 47 kDa, respectively, and are not functionally identical. The two GSK-3 isoforms have a unique glycine-rich N-terminal domain (where Ser 21/9 is found) and a conserved (98% homology) kinase domain (Tyr 279/216). GSK-3 is normally active in cells and is unusual in that it is regulated through inhibition. Although, originally found to play a pivotal role in glycogen metabolism, it has since been realised that GSK-3 has far more numerous signalling roles, including the Hedgehog pathway, Wnt/ β -catenin pathway and NFAT inactivation (see reviews (Cohen and Frame, 2001; Doble and Woodgett, 2003; Dominguez and Green, 2001; Frame and Cohen, 2001; Harwood, 2001)).

Phospholipase D (PLD)

Phospholipase D (PLD) is activated in response to various chemokines (Bacon et al., 1998) and is involved in vesicular protein transport and may additionally play a part in actin

polymerisation that is thought to be required for cell movement. Recent evidence suggests a link between PI3K γ and PLD (Toda et al., 1999). ARF-GEFs (ADP-ribosylation factor guanine-nucleotide-exchange factors) such as Arno, Grp-1 and cytohesin contain PH domains that bind D3 PIs on the plasma membrane and may then activate ARFs (ADP-ribosylation factors), in particular ARF6, which in turn leads to PLD activation and subsequent phosphatidic acid accumulation and actin reorganisation (fig. 1.25). See the reviews by (Liscovitch et al., 1999; Turner and Brown, 2001) for more detail.

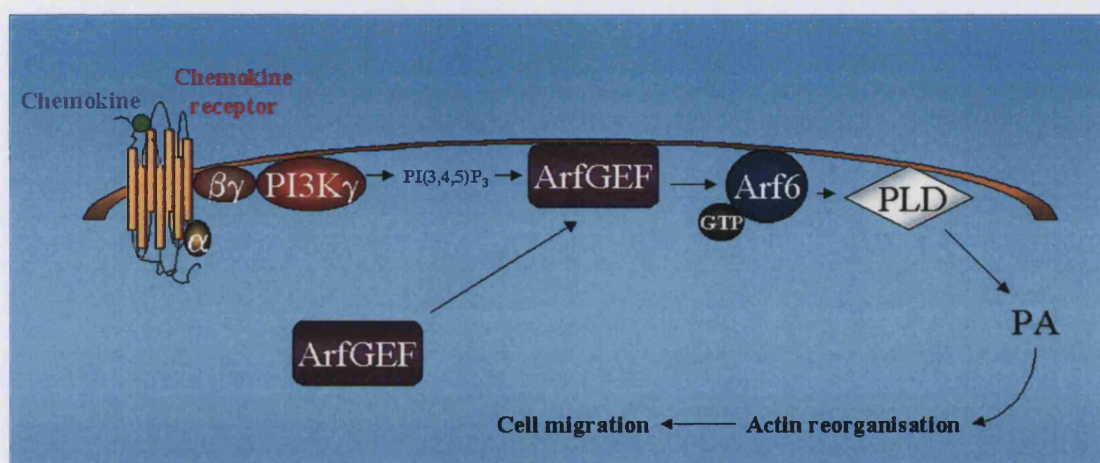


Figure 1.25. The potential interactions between PI3K, PLD and actin rearrangement upon chemokine receptor ligation. Reproduced from (Sotsios and Ward, 2000). Abbreviations: Arf6, ADP-ribosylation factor 6; ArfGEF, ADP-ribosylation factor guanine-nucleotide-exchange factor; PA, phosphatidic acid.

Regulation of PI3K-Dependent Signalling: SHIP and PTEN

Both positive and negative regulatory signalling cascades are vital for homeostasis during cell activation. The activity of PI3K is opposed by the inositol 3-lipid phosphatase PTEN (Phosphatase and tenisn homologue deleted on chromosome ten) and more indirectly by the inositol 5-phosphatase SHIP (SH2-containing inositol (poly)phosphate 5-phosphatase) (fig. 1.20). *PTEN* is a human suppressor gene for which this status is supported by numerous lines of evidence. *PTEN* was first identified in 1997 at chromosome 10q23 and encodes the 403 amino acid PTEN protein that is a member of the protein tyrosine phosphatase family

(Leslie and Downes, 2002; Seminario and Wange, 2002). This chromosomal region has been found to be deleted in many tumour types and, after p53, has been found to be the most highly mutated gene in human cancers (Cairns et al., 1997; Li et al., 1997; Risinger et al., 1997; Steck et al., 1997; Tashiro et al., 1997). This additionally highlights the importance of PI3K in mammalian cells along with the data from PTEN^{-/-} mice that die during embryonic development (Di Cristofano et al., 1998). PTEN (also known as MMAC1 and TEP1) is responsible for dephosphorylating several phosphoinositide signalling molecules *in vitro*, specifically removing the phosphate group from the D3 position of the inositol ring. PI(3,4)P₂ and PI(3,4,5)P₃ are the most efficient substrates and this is further highlighted in cells lacking PTEN that display elevated levels of these lipids (Astoul et al., 2001; Cantrell, 2001).

The SHIP (110 – 145 kDa) family of proteins is responsible for dephosphorylating the D5 position of the inositol ring and its major substrate is PI(3,4,5)P₃. There are currently three SHIP1 isoforms (α,β,δ) and one SHIP2 (160kDa). Loss of SHIP results in an unbalanced immune response and culminates in the development of autoimmunity (for more detail see (March and Ravichandran, 2002)).

Although PTEN and SHIP regulate the products of activated PI3K, it is also necessary to terminate the action of the kinase itself. This requires the dephosphorylation of the tyrosine residues recognised by the SH2 domains of the regulatory p85 subunit of PI3K (class I). This process may require SHP-1 (SH2-containing phosphatase-1) as this phosphatase has been shown to bind PI3K and can also dephosphorylate Tyr 688 of p85 to further inactivate PI3K (Cuevas et al., 2001).

The Extracellular Regulated Kinase-Mitogen Activated Protein Kinase Pathway (ERK-MAPK)

In addition to the PI3K pathway, the mitogen-activated protein kinase pathways have been intensely studied over the years, and especially the extracellular-regulated kinase (ERK)-MAPK pathway. The activation of a MAPK involves a three kinase cascade consisting of a MAP kinase kinase (MAPKKK, MKKK or MEKK) which activates a MAP/ERK kinase

(MAPKK, MKK or MEK), which then stimulates a phosphorylation-dependent increase in the activity of the MAPK (e.g. ERK), and the subsequent phosphorylation of a variety of intracellular targets, including transcription factors, transcriptional adaptor proteins, membrane and cytoplasmic substrates, and other kinases (fig. 1.26).

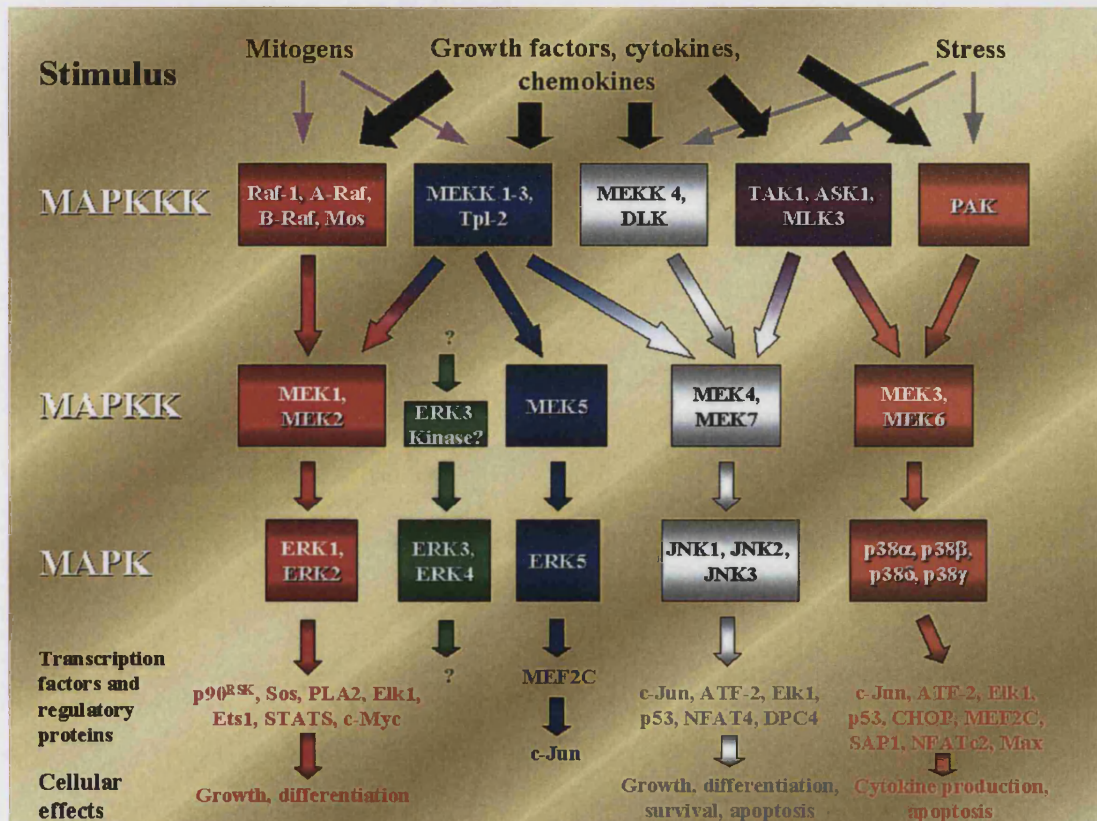


Figure 1.26. The major MAPK signalling modules. The MAPK module is composed of a MAPKKK, MAPKK, and a MAPK. Each pathway can be activated with a variety of stimuli, with the MAPKKs being activated by one or more MAPKKKs, but MAPKKs are relatively specific for their activation of target MAPKs (modified from (Garrington and Johnson, 1999; Rincon, 2001)).

The MAPK family consists of both stress-activated (SAPK) and mitogen-activated kinases and there are currently three major groups of MAPK modules: the p38 Map kinase family (4 isoforms - α , β , δ , γ), the classical ERK family (isoforms ERK1/2, but 6 others identified, ERK3-8, and are involved in different pathways/modules), and the c-Jun NH₂-terminal kinase (JNK) family (3 isoforms – JNK1/2/3) (Platanias, 2003). Each MAPK is

activated by dual phosphorylation on a Thr-Xaa-Tyr motif by upstream kinases (e.g. Thr 202/Tyr 204 for ERK1 by MEK1/2 – ERK1/2 is the only known substrate of MEK1/2). Despite the wide use of ERK1/2, p38 and JNK phospho-antibodies in cell signalling (and largely used as an indicator of receptor activation), very little detail is known about how they are regulated by the MAPKKs. MAPKs phosphorylate their substrates on serine or threonine residues which precede a proline but specificity *in vivo* is further enhanced by the presence of distinct docking sites. The MAPKs are ubiquitously expressed and activated by a wide variety of stimuli, including chemokines, growth factors, cytokines and, as their name suggests, mitogens and cellular stress (p38 and JNK) (Arndt et al., 2003; Bonello and Khachigian, 2003; Sodhi and Sethi, 2003; Uchida et al., 2003; Wuyts et al., 2003). The MAPK family phosphorylate MAPK-activated protein (MAPKAP) kinases, which can be subdivided into two groups: two kinase domains within a single polypeptide and the single kinase domain proteins. Two kinase domain enzymes are MAPKAP-K1 (more commonly known as p90^{RSK}) – involved in processes such as cell survival, gene transcription and meiosis, and mitogen and stress-activated protein kinase (MSK) – possible role in gene transcription. The single kinase domain MAPKAPs are MAPKAP-K2, MAPKAP-K3, MAPKAP-K5 (also known as PRAK (p38-regulated/activated kinase)), and MAPK-integrating kinase (MNK) (Garrington and Johnson, 1999; Marinissen and Gutkind, 2001; Rincon, 2001; Rincon et al., 2001; Widmann et al., 1999).

Following chemokine-induced G protein heterotrimeric dissociation, the G α subunit is released in addition to the $\beta\gamma$ subunits. The G α subunit does not appear to be involved in cell migration events but does bind to and activate Src, and possibly other related, tyrosine kinases. This provides a possible means of activating the ERK-MAPK pathway via the Ras-Raf pathway (reviews see (Gutkind, 2000; Kerkhoff and Rapp, 2001)). Another possible route is dependent on the G $\beta\gamma$ subunit activating the PKC (and may involve the ζ or δ isoforms) pathway and then the Raf-MEK-ERK pathway (this maybe Ras independent (Faure et al., 1994) and coupled to a G α_q subunit) (Bonacchi et al., 2001; Smit et al., 2003; Tilton et al., 2000). The activation of ERK may also involve the PI3K pathway, and in particular PI3K's intrinsic protein kinase activity, as some studies have shown that PI3K inhibitors can block ERK phosphorylation in response to chemokine receptor ligation (Knall et al., 1996; Sotsios et al., 1999; Sullivan et al., 1999b) (fig. 1.27). The other two

major MAPK pathways, p38 and JNK, have also been described to lie downstream of chemokine receptors (Merz et al., 2003; Mocsai et al., 2003; Sodhi and Biswas, 2002; Vlahakis et al., 2002).

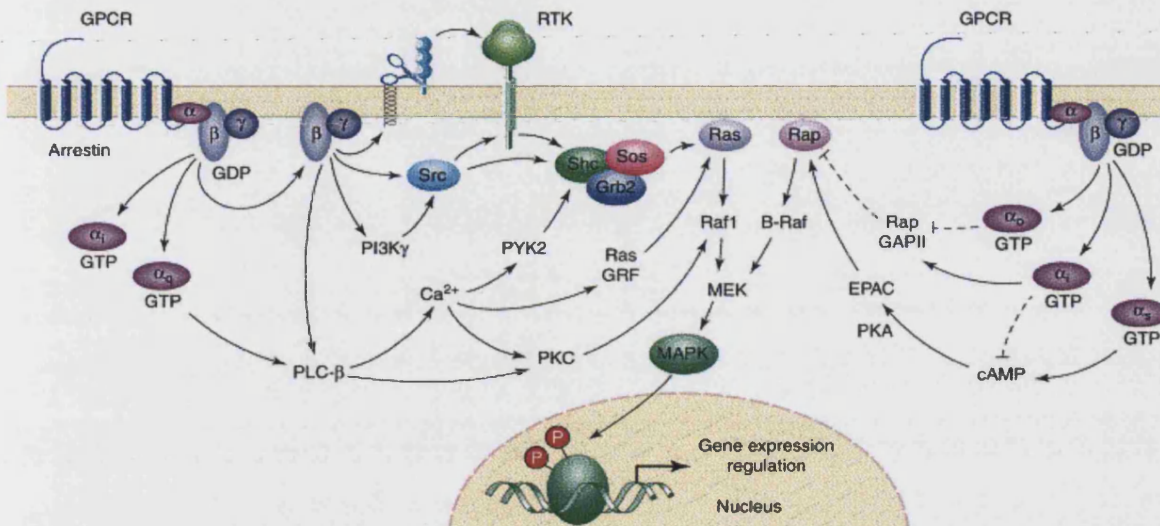


Figure 1.27. Possible pathways linking chemokine receptors to ERK-MAPK activation. Biochemical routes initiated by $\beta\gamma$ -subunits can stimulate Ras by the activation of receptor and non-receptor tyrosine kinases, which results in the recruitment of Sos to the membrane and the exchange of GDP for GTP bound to Ras. Activated $G_{\alpha q}$ can stimulate Raf1 through protein kinase C (PKC) or by stimulating Ras by the Ca^{2+} -dependent activation of RasGRF and tyrosine kinases acting on Sos. $G_{\alpha i}$, $G_{\alpha o}$ and $G_{\alpha s}$ can also use tissue-restricted pathways regulating Rap, which can stimulate B-Raf and lead to the activation of MAPK. Activated MAPK translocates to the nucleus and phosphorylates nuclear proteins, including transcription factors, thereby regulating gene expression. Arrows represent positive stimulation and broken lines represent inhibition (see text for more details). *Abbreviations:* EPAC, exchange protein activated by cAMP; GAP, GTPase-activating protein; GRF, guanine-nucleotide releasing factor; MEK, MAPK kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PLC, phospholipase C; RTK, receptor tyrosine kinase. Figure acquired from (Marinissen and Gutkind, 2001).

The ROCK and Rho Lifestyle of Chemokine Receptors

A couple of recent studies have suggested that the PI3K pathway may not participate in T lymphocyte migration and that there may be differences in the mechanisms of cell migration between lymphocytes (do not require PI3K) and myeloid cells (require PI3K) (Cinamon et al., 2001; Smit et al., 2003). A pathway that has gained prominence recently for its

apparent necessity for chemokine-mediated lymphocyte migration is the Rho-ROCK (Rho-associated coiled-coil forming kinase) pathway (Bardi et al., 2003; Vicente-Manzanares et al., 2002).

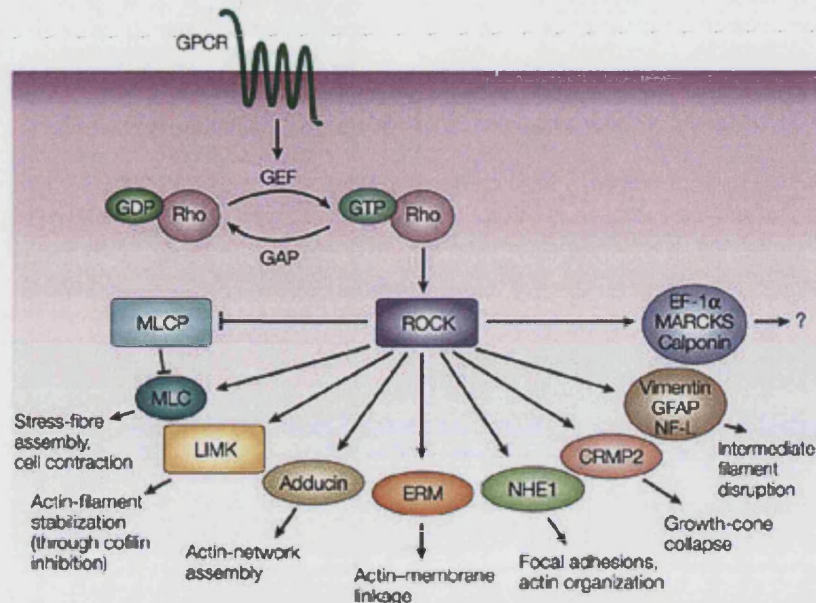


Figure 1.28. Rho-ROCK signalling pathways. Rho activation of ROCKs leads to the phosphorylation of several substrates, resulting in various cellular responses. In particular, phosphorylation of the myosin binding subunit of myosin light chain phosphatase (MLCP) by ROCKs inhibits the dephosphorylation of myosin light chain (MLC), thereby increasing myosin II activity and stress-fibre formation and contractility (Riento and Ridley, 2003). *Abbreviations:* CRMP2, collapsin response mediator protein-2; EF-1 α , elongation-factor 1 α ; ERM, ezrin-radixin-moesin; GAP, GTPase activating protein; GFAP, glial fibrillary acidic protein; LIMK, LIM (for Lin11, Isl1 and Mec3) kinase; MARCKS, myristylated alanine-rich C-kinase substrate; NHE1, Na-H exchanger-1; NF-L, neurofilament protein.

Rho is a family of small GTPases that were initially cloned on the basis of their similarity with the Ras oncogenes. There are currently 18 members that can be further subdivided depending upon sequence similarity and biological function and include groups Rac1, cdc42 and RhoA (Sahai and Marshall, 2002). Rho family GTPases cycle between GDP-bound inactive and GTP-bound active forms, with GTP binding promoted by Rho-guanine nucleotide exchange factors (Rho-GEFs) and GTP hydrolysis catalysed by Rho-GTPase activating proteins (Rho-GAPs) (Fukata et al., 2003; Moon and Zheng, 2003). Rho-family

GTPases have been observed to regulate the actin cytoskeleton and cell adhesion, and have numerous effector molecules (see reviews (Aspenstrom, 1999a; Aspenstrom, 1999b)). One such downstream effector of the RhoA, RhoB and RhoC GTPases is the ROCK serine/threonine protein kinase. To date there have been 2 ubiquitously expressed ROCK isoforms identified, ROCK1 and ROCK2, that are ~ 160 kDa in size. ROCK kinases have a plethora of target substrates that contain the motif R/K-Xaa-S/T or R/K-Xaa-Xaa-S/T, including myosin light chain (MLC) – the major substrate of the Rho-ROCK pathway – and thus participates in actomyosin contractility (fig. 1.28) (for in depth reviews of ROCK read (Amano et al., 2000; Riento and Ridley, 2003)).

1.6 Aims of Study

Published research into the signalling pathways activated upon CCR4 ligation is very meagre, and there are currently no dedicated papers on this matter, with only a few papers demonstrating a calcium flux upon CCR4 ligation and PTX-sensitive migration (Andrew et al., 1998; Inngjerdingen et al., 2000; Oh et al., 2002; Suzuki et al., 1999). This is in stark contrast to receptors such as CXCR3, and especially CXCR4, in which there are a number of published signalling papers (Ganju et al., 1998a; Ganju et al., 2000; Wain et al., 2002).

The aim of this study is to start to elucidate the signal transduction pathways that exist downstream of CCR4 upon its ligation with its chemokine ligands, MDC and TARC, and more importantly, those pathways that are required for CCR4-mediated directed T cell migration. It is apparent from the preceding overview of chemokine function and signalling that this family of GPCRs have a pivotal role to play in the general immunity of the human organism. Thus any further knowledge of how these chemokines regulate cell migration will expectantly provide useful information into these processes and facilitate the identification of future therapeutic targets within this field, and more specifically with relation to CCR4 (research in this area being highly underdeveloped). Specifically, the project will focus investigations in the following areas:

- The role of PI3K in CCR4-mediated signalling and function.
- Examination of the calcium pathway downstream of ligated CCR4 and the role of intracellular calcium mobilisation in directed cell migration.
- Investigate the mediators that are required for activation of the ERK-MAPK and PKB pathways.
- Elucidate the CCR4-activated signalling cascades that are critical for chemotaxis.

Since this study is primarily concerned with the activity of CCR4 on T cells, the majority of the experiments will be performed in leukaemic T cell lines (principally the CEM T cell line) and in more physiologically 'normal' T cells (i.e. T lymphoblasts and *in vitro* generated human Th2 cells). This will also allow for a comparison of activities between the transformed and non-transformed cells.

2

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1 Materials

Antibodies

<i>Antibody</i>	<i>Species</i>	<i>Monoclonal or Polyclonal</i>	<i>Supplier</i>
Anti-CCR4 PE conjugated	Mouse	Monoclonal	BD PharMingen, Oxford, UK
Anti-CD28 (clone 9.3)	Mouse	Monoclonal	Carl June, Uni. Of Pennsylvania, USA
Anti-CD3 (clone UCHT1) for Th2 cell polarisation	Mouse	Monoclonal	BD Pharmingen, Oxford, UK
Anti-CD3 (clone UCHT1)	Mouse	Monoclonal	Doreen Cantrell, School of Life Sciences, Dundee, UK
Anti-ERK 1/2	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-goat Ig (HRP-conjugated)	Rabbit	Polyclonal	DAKO, Glostrup, Denmark
Anti-GSK-3 α/β	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-hCD95L	Goat	Polyclonal	R & D Systems, Abingdon, UK
Anti-IFN γ	Goat	Polyclonal	R & D Systems, Abingdon, UK
Anti-IL-12	Goat	Polyclonal	R & D Systems, Abingdon, UK
Anti-mouse Ig (HRP-conjugated)	Rabbit	Polyclonal	DAKO, Glostrup, Denmark
Anti-p110 δ	Goat	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-p110 γ (clone H-199)	Goat	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-p85	Rabbit	Polyclonal	Bart Vanhaesebroeck, Ludwig Institute, London, UK
Anti-phospho ERK 1/2	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-phospho GSK-3 α/β	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-phospho p38	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-phospho p90 ^{RSK}	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-phospho PKB ³⁰⁸	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-phospho PKB ⁴⁷³	Rabbit	Polyclonal	NEB (UK) Ltd, Hitchin, UK
Anti-phosphotyrosine (clone 4G10)	Mouse	Monoclonal	Upstate Biotechnology, NY, USA
Anti-PI3K C2 α	Rabbit	Polyclonal	Bart Vanhaesebroeck, Ludwig Institute, London, UK

Anti-PI3K C2 β	Rabbit	Polyclonal	Bart Vanhaesebroeck, Ludwig Institute, London, UK
Anti-PKB	Goat	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-PKC δ	Rabbit	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-PTEN	Rabbit	Polyclonal	Santa Cruz Biotechnology, Santa Cruz, USA
Anti-Pyk2	Rabbit	Polyclonal	Upstate Biotechnology, NY, USA
Anti-rabbit Ig (HRP-conjugated)	Goat	Polyclonal	DAKO, Glostrup, Denmark
Anti-SHIP	Mouse	Monoclonal	Santa Cruz Biotechnology, Santa Cruz, USA
PE-conjugated IgG ₁ , κ	Mouse	Monoclonal	BD Pharmingen, Oxford, UK

Consumables

<i>Consumable</i>	<i>Supplier</i>
0.2 μ m syringe filters	Fisher Scientific, Loughborough, UK
0.2 ml thin-walled PCR tubes with lids	Promega, Southampton, UK
0.5 ml Eppendorff centrifuge tubes	NUNC, UK
1.5 ml Eppendorff centrifuge tubes	NUNC, UK
10 ml Disposable Pipettes	NUNC, UK
30 ml Disposable Pipettes	NUNC, UK
60 & 100 ml pots	Sterilin, UK
96-well plates for chemotaxis	Greiner-bione, Germany
Black-walled, clear bottom 96-well plates, poly-D-lysine coated	Corning Costar UK Ltd, High Wycombe, UK
Butterfly needles	Terumo, Belgium
ChemoTx disposable chemotaxis plates	NeuroProbe Inc, Gaithersburg, MD, USA
Conical 15 ml centrifuge tubes	Becton Dickinson, USA
Conical 50 ml centrifuge tubes	Becton Dickinson, USA
Cryovials 2ml	Fisher Scientific, Loughborough, UK
Filter paper	Whatman, Maidstone, UK
Gel loading tips 100 μ l	Fisher Scientific, Loughborough, UK
Gilson pipette tips (10, 200, 1000, 5000 μ l)	Fisher Scientific, Loughborough, UK
Glass cover slips (diameter 23 mm)	Fisher Scientific, Loughborough, UK
Glass slides	Fisher Scientific, Loughborough, UK
Haemocytometer	Fisher Scientific, Loughborough, UK
Nitrocellulose blotting membrane	Whatman, Maidstone, UK
Parafilm	Fisher Scientific, Loughborough, UK
Pastettes 1 & 3ml	Alpha Lab, Hampshire, UK
Petri dishes (tissue culture)	NUNC, UK

Polypropylene FACS tubes	Becton Dickinson, USA
PVP-free polycarbonate 5 µm filters	NeuroProbe Inc, Gaithersburg, MD, USA
Silica Gel TLC plates, 19 lanes	Whatman, Maidstone, UK
Starsted screw-lid 2 ml centrifuge tubes	Starsted, Germany
Sterile syringes 5, 20 & 60 ml	Fisher Scientific, Loughborough, UK
Tissue culture 12-well plates	NUNC, UK
Tissue culture 6-well plates	NUNC, UK
Tissue culture flasks (175 cm ²)	NUNC, UK
Tissue culture flasks (25 cm ²)	NUNC, UK
Tissue culture flasks (80 cm ²)	NUNC, UK
Tissue culture flasks (triple 175 cm ²)	NUNC, UK
Xomat Kodak flim	Kodak, UK

Reagents

<i>Reagent</i>	<i>Supplier</i>
[γ- ³² P] ATP	Amersham, Little Chalfont, UK
30% Bis-Acrylamide	BioRad, UK
Acetone	Fisher Scientific, Loughborough, UK
Adenosine triphosphate (ATP)	Sigma Aldrich, Gillingham, UK
Agarose (molecular biology grade)	Sigma Aldrich, Gillingham, UK
Ammonium persulphate (APS)	BDH, UK
Ammonium phosphate (NH ₄) ₂ PO ₄	Sigma Aldrich, Gillingham, UK
Aprotinin	Sigma Aldrich, Gillingham, UK
Blue Juice TM loading buffer	Intitrogen, Paisley, UK
Boric acid	Sigma Aldrich, Gillingham, UK
Bovine serum albumin (BSA)	Sigma Aldrich, Gillingham, UK
Brilliant black	Sigma Aldrich, Gillingham, UK
Bromophenol blue	BDH, UK
Butanol	Fisher Scientific, Loughborough, UK
Calcein-AM	Molecular Probes, Eugene, OR, USA
Calcium chloride CaCl ₂	Sigma Aldrich, Gillingham, UK
CD4 ⁺ microbeads	Miltenyi Biotec, UK
CD45RA ⁺ microbeads	Miltenyi Biotec, UK
CellTiter 96 AQueous ONE solution	Promega, Southampton, UK
Chloroform CHCl ₃	Fisher Scientific, Loughborough, UK
Deoxynucleoside triphosphates: dATP, dCTP, dGTP, and dTTP	Boehringer Mannheim, Germany
Digitonin	BDH, UK
Dimethyl sulphoxide (DMSO)	Sigma Aldrich, Gillingham, UK
Dissociation buffer	Life Technologies, Paisley, UK
DNase I	Promega, Southampton, UK
DTT (dithiothreitol)	Promega, Southampton, UK

EDTA	Sigma Aldrich, Gillingham, UK
EGTA	Sigma Aldrich, Gillingham, UK
Enhanced chemoilluminescence (ECL)	Amersham, Little Chalfont, UK
Ethanol (EtOH)	Fisher Scientific, Loughborough, UK
Ethidium bromide	BioRad, Hemel Hempstead, UK
Ethyl formate	Fisher Scientific, Loughborough, UK
Expand TM High Fidelity PCR System	Boehringer Mannheim, Germany
Flo-scint IV scintillation fluid	Packard Bioscience, Groningen, Netherlands
Fluo-4, AM	Molecular Probes, Eugene, OR, USA
Foetal bovine serum (FBS)	Life Technologies, Paisley, UK
Folch lipids from bovine brain	Sigma Aldrich, Gillingham, UK
Fura-2 AM	Molecular Probes, Eugene, OR, USA
Geneticin (G418 sulphate)	BDH, UK
Glacial acetic acid	Fisher Scientific, Loughborough, UK
Glucose	Fisher Scientific, Loughborough, UK
Glutaraldehyde	Sigma Aldrich, Gillingham, UK
Glutathione sepharose beads	Sigma Aldrich, Gillingham, UK
Glycerol	Fisher Scientific, Loughborough, UK
Glycine	Sigma Aldrich, Gillingham, UK
HBSS	Life Technologies, Paisley, UK
HEPES 1 M solution	Sigma Aldrich, Gillingham, UK
HPLC Partisphere SAX column	Whatman, Maidstone, UK
Hydrochloric acid (HCl)	BDH, UK
Hygromycin B	Calbiochem, Nottingham, UK
Insulin-transferrin-selenium (ITS) 100x	Life Technologies, Paisley, UK
Interleukin-2 (IL-2)	Chemicon, Hampshire, UK
Interleukin-4 (IL-4)	Novartis Pharmaceuticals, UK
Ionomycin	Calbiochem, Nottingham, UK
Ladder 15 blunt end fragments 100 base pair (100 – 1500 bp scale)	GIBCO BRL, Paisley, UK
Lauryl sulphate (SDS)	Sigma Aldrich, Gillingham, UK
Leupeptin	Sigma Aldrich, Gillingham, UK
L-glutamine	Life Technologies, Paisley, UK
Lithium chloride (LiCl ₂)	Sigma Aldrich, Gillingham, UK
Lymphoprep (Ficoll-paque 1.077 g/ml density)	Nycomed Pharma AS, Oslo, Norway
Magnesium chloride (MgCl)	Sigma Aldrich, Gillingham, UK
Marvel (fat-free dry milk)	Supermarket (Fresh Store, Uni. Bath)
MEM vitamins	Life Technologies, Paisley, UK
Mercaptoethanol	Sigma Aldrich, Gillingham, UK
Methanol (MeOH)	Fisher Scientific, Loughborough, UK
Methylamine (25-30% in H ₂ O)	Fisher Scientific, Loughborough, UK
Molecular weight marker (for WB)	Life Technologies, Paisley, UK
NP-40 (IGEPAL)	Fisher Scientific, Loughborough, UK
oligo(dT) ₁₂₋₁₈	Invitrogen, Paisley, UK

Orthophosphoric acid [^{32}P] 5 mCi/ml	Amersham, Little Chalfont, UK
Paraformaldehyde	Sigma Aldrich, Gillingham, UK
Penicillin/Streptomycin (P/S) 100x	Life Technologies, Paisley, UK
Pepstatin A	Sigma Aldrich, Gillingham, UK
Petroleum ether (B.P. 40-60 °C)	BDH, UK
Phenylmethyl sulphonyl fluoride (PMSF)	Sigma Aldrich, Gillingham, UK
Phorbol 13-myristate 12-acetate (PMA)	Calbiochem, Nottingham, UK
Phosphate buffered saline (PBS)	Sigma Aldrich, Gillingham, UK
Phosphoric acid	Fisher Scientific, Loughborough, UK
Pluronic acid F-127	Molecular Probes, Eugene, OR, USA
Poly-L-lysine	Sigma Aldrich, Gillingham, UK
Ponceau S solution	Sigma Aldrich, Gillingham, UK
Potassium acetate	Sigma Aldrich, Gillingham, UK
Potassium oxalate	Sigma Aldrich, Gillingham, UK
Probenecid	Sigma Aldrich, Gillingham, UK
Propan-1-ol	Fisher Scientific, Loughborough, UK
Propan-2-ol	Fisher Scientific, Loughborough, UK
Protein A sepharose	Sigma Aldrich, Gillingham, UK
Protein G sepharose	Pharmacia, UK
Recombinant human MDC (rhMDC)	R & D Systems, Abingdon, UK
Recombinant human SDF-1 (rhSDF-1)	R & D Systems, Abingdon, UK
Recombinant human TARC (rhTARC)	R & D Systems, Abingdon, UK
RNase AWAY TM reagent	Invitrogen, Paisley, UK
Rnase-free (nuclease-free) water	Promega, Southampton, UK
RNasin – a non-competitive ribonuclease inhibitor 49.8 kDa recombinant E.coli	Promega, Southampton, UK
Sodium azide	Sigma Aldrich, Gillingham, UK
Sodium chloride (NaCl)	Sigma Aldrich, Gillingham, UK
Sodium Fluoride (NaF)	Sigma Aldrich, Gillingham, UK
Sodium hydroxide (NaOH)	BDH, UK
Sodium orthovanadate	Sigma Aldrich, Gillingham, UK
Staphylococcal enterotoxin B (SEB)	Sigma Aldrich, Gillingham, UK
Superscript II RNase H ⁻ Reverse Transcriptase	Promega, Southampton, UK
TEMED	Sigma Aldrich, Gillingham, UK
Tetrabutyl ammonium hydrogen sulphate (TBAS)	Sigma Aldrich, Gillingham, UK
Tris	Sigma Aldrich, Gillingham, UK
Trypan blue	Sigma Aldrich, Gillingham, UK
Trypsin-EDTA	Life Technologies, Paisley, UK
Tween-20	Sigma Aldrich, Gillingham, UK
Versene	Life Technologies, Paisley, UK

Cells and Media

<i>Cells or Media</i>	<i>Supplier</i>
CEM cells	Dr Z. Brown, Novartis, Horsham, UK
Chinese hamster ovary (CHO) K1 cells	Dr Z. Brown, Novartis, Horsham, UK
CHO-CCR4 ⁺ cells	Dr Z. Brown, Novartis, Horsham, UK
DMEM	Life Technologies, Paisley, UK
MOLT-4 cells	ECACC, Poole, Dorset, UK
HUT-78 cells	Dr Z. Brown, Novartis, Horsham, UK
Jurkat cells (J6 clone)	Dr. A. Alcover, INSERM, Paris, France
RPMI 1640	Life Technologies, Paisley, UK
RPMI 1640 w/o phenol red	Life Technologies, Paisley, UK

Inhibitors and other Pharmacological Tools

<i>Pharmacological Tool</i>	<i>Supplier</i>
2-APB	Calbiochem, Nottingham, UK
8-Br-cADPR	Sigma Aldrich, Gillingham, UK
AG490	Calbiochem, Nottingham, UK
AG9	Calbiochem, Nottingham, UK
Caffeine	Calbiochem, Nottingham, UK
Dantrolene	Calbiochem, Nottingham, UK
Gö6976	Calbiochem, Nottingham, UK
L-NAME	Calbiochem, Nottingham, UK
LY294002	Calbiochem, Nottingham, UK
Nifedipine	Calbiochem, Nottingham, UK
PD98059	Calbiochem, Nottingham, UK
Pertussis toxin (PTX)	Sigma Aldrich, Gillingham, UK
PKB inhibitor I (PKBi)	Tocris Pharmaceuticals
RO-32-0432	Calbiochem, Nottingham, UK
Rottlerin	Calbiochem, Nottingham, UK
Ryanodine	Calbiochem, Nottingham, UK
SB202190	Calbiochem, Nottingham, UK
SB203580	Calbiochem, Nottingham, UK
TLCK	Calbiochem, Nottingham, UK
TPCK	Calbiochem, Nottingham, UK
U73122	Calbiochem, Nottingham, UK
U73343	Calbiochem, Nottingham, UK
WHI-P154	Calbiochem, Nottingham, UK
Wortmannin	Calbiochem, Nottingham, UK
Y27632	Calbiochem, Nottingham, UK

2.2 Methods

Cell Culture

The following cell lines were used in this study (descriptions from ECACC or ATCC websites):

- CEM – human Caucasian acute T lymphoblastoid leukaemia cell
- Jurkat J6 – Leukaemic T lymphoblast cell
- Hut-78 – Leukaemic T lymphoblast cell
- CHOK1 – Chinese hamster ovary cell
- CHOK1hCCR4 – Chinese hamster ovary cell stably transfected with a plasmid coding to generate functional human CCR4 receptors on its surface

The following cells were generated from peripheral blood mononuclear cells:

- T lymphoblasts (T blasts) – super antigen-activated T cells
- Th2 lymphocytes – T helper 2-like cells

Suspension Cells

All suspension cell lines (Jurkat, CEM, HUT-78) were cultured in humidified incubators in 5 % CO₂ at 37 °C in RPMI 1640 medium, supplemented with 10 % (v/v) FBS, 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium). Cells were passaged when a concentration of approximately 1-2 x 10⁶ cells/ml was reached. Cells were resuspended in RPMI 1640 with 0.1 % BSA for 1 hour prior to use in experiments (unless stated otherwise).

Adherent Cells

CHOK1 and CHOK1hCCR4 cells were cultured in humidified incubators at 37 °C, 5 % CO₂ and maintained in DMEM (Dulbecco's Modified Eagles Medium) supplemented with 10 % (v/v) foetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.1 µM sodium selenite, 4 mg/ml L-proline, 2 mM L-glutamine and, for selection purposes, 200 µg/ml hygromycin and 1mg/ml geneticin (complete medium). Cells were passaged when fully confluent. For experiments, CHOK1 and CHOK1hCCR4 confluent cells were washed, seeded at required density in 1.5 ml centrifuge tubes (in most cases) and incubated (37 °C water bath) in fresh medium without foetal bovine serum but with 0.1 % BSA for 1 hour before stimulation. Growth-arrested cells were treated with the appropriate concentrations of stimuli in foetal bovine serum-free medium + 0.1 % BSA.

Cell Counting

Cells were resuspended in 50 ml media (but this varied depending on the suspected number of cells present) and a 100 µl sample removed and aliquoted in to a 0.5 ml microcentrifuge tube containing 100 µl 0.4 % trypan blue (this checks for cell viability – it is a negatively charged chromophore (contains two azo chromophores) and will not react with the cell unless the cell membrane is damaged. A damaged cell results in the dye binding cellular proteins and thereby staining the cell blue). Non-viable cells are omitted from the counting process – there ideally should not be any more than 5 % of total cells that are non-viable. The tube was gently mixed and ~10 µl of the mixture was transferred to one side of a haemocytometer. The number of viable cells in one corner square (comprising 16 smaller squares) was counted using a light microscope (fig. 2.1).

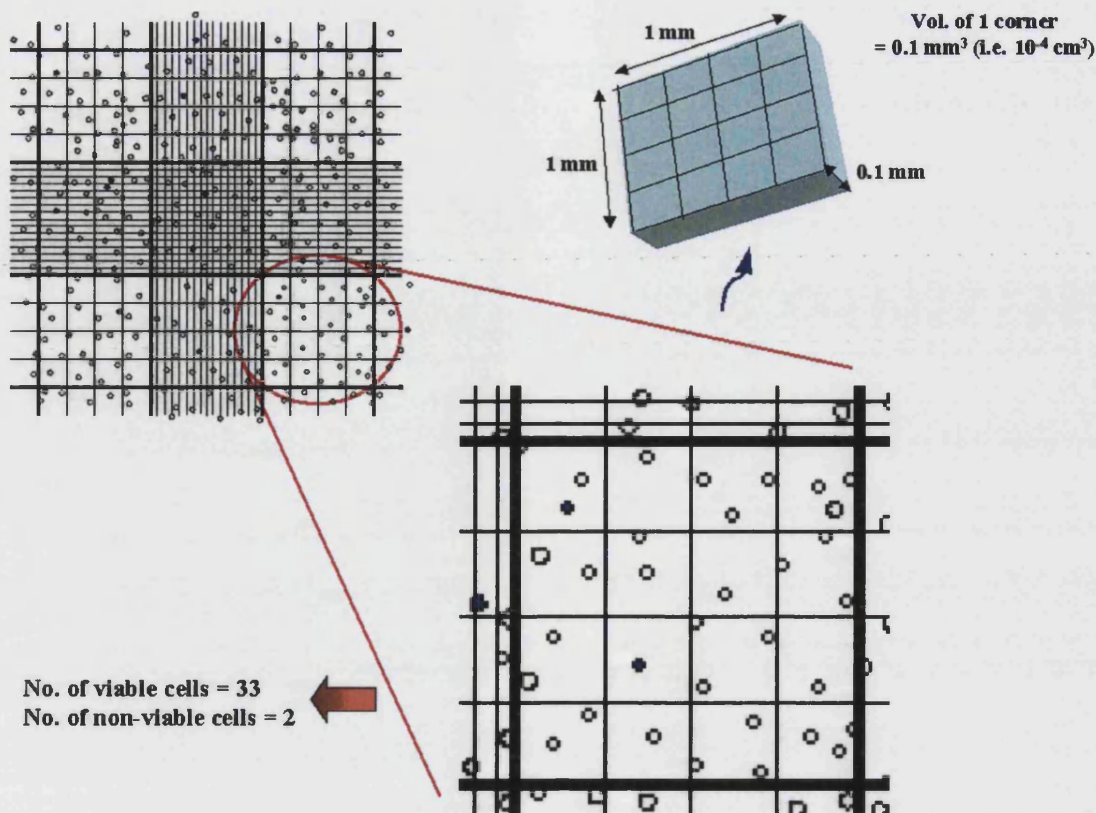


Figure 2.1. Cell counting using a standard haemocytometer. Those cells touching the bottom and bottom right outside lines are counted, whereas those touching the top and top left outside lines are excluded. This gives the number of viable cells counted as 33 (with 2 non-viable cells).

The number of cells counted should be over 100 and if this was not the case then another corner square was counted. The number of cells/ml was calculated using the formula below:

$$\text{Cells/ml} = (\text{average count per square}) \times (\text{dilution factor}) \times 10^4 \text{ [chamber conversion factor]}$$

Therefore, for example in figure 2.1 (using dilution factor described in text):

$$\begin{aligned} &= 33 \times (50 \times 2) \times 10^4 \\ &= 3.3 \times 10^7 \text{ cells/ml} \\ &(\text{cell viability } [3.3 \times 10^7] / [3.5 \times 10^7] \times 100 = 94.3 \%) \end{aligned}$$

Freezing Cells

Cells are frozen in order to provide a stock of cells of a particular age and passage number thereby minimising the transformation of cell lines that may occur with long term cell culture, and thus providing batches of cells that are similar for all experiments that are performed. Cells were grown to an appropriate density (i.e. $1\text{--}2 \times 10^6$ for suspension cells or confluent for adherent cells), washed three times in appropriate media without supplements (wash media), and counted using a haemocytometer and the stain trypan blue. Trypan blue is a stain (a negatively charged chromophore) that checks cells for their viability and will only enter cells whose membranes are damaged. Cells were resuspended at a concentration of $2\text{--}10 \times 10^6$ cells/ml in 'freeze media' (90 % FBS and 10 % DMSO) and 1 ml volumes aliquoted into cryovials. The cryovials were then placed into a 'Mr Frosty' (filled with the required volume (250 ml) of isopropanol (i.e. propan-2-ol), at room temperature, and then placed into a $-80\text{ }^{\circ}\text{C}$ freezer. The 'Mr Frosty' ensures a freezing rate of $1\text{ }^{\circ}\text{C}/\text{min}$, thereby reducing the occurrences of artifacts. The following day (16 – 24 hours later) the cryovials were transferred into canes and placed into liquid nitrogen tanks for long-term storage.

Thawing Cells

Appropriate wash media and media with 20 % FBS (instead of normal 10 %), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin were warmed to $37\text{ }^{\circ}\text{C}$ in a water bath. Cryovials containing the desired cells were recovered from liquid nitrogen and thawed immediately by partial submersion in a $37\text{ }^{\circ}\text{C}$ water bath for 2 – 3 minutes, with regular agitation. Cells were then transferred to 50 ml conical tubes containing pre-warmed wash media and gently mixed and centrifuged at 1500 rpm for 5 minutes at rtp. Cells were washed a further two times, then resuspended in media containing 20 % FBS (volume of re-suspension depends on number of cells, but generally 10 – 50 ml) and transferred to an appropriate size tissue culture flask (25 cm^2 flask for ~ 10 mls, 80 cm^2 flask for ~ 30 mls, and 175 cm^2 flask for $\sim 50+$ ml). Over the following days cells were assessed and, once optimal density reached,

media volume was expanded with cells transferred into larger flasks and resuspended in complete media (i.e. media with selective reagents if required).

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique used to amplify virtually any DNA segment that lies between two regions of known sequence. The idea of PCR was conceived by Kary Mullis (Nobel Laureate 1993) in 1983 at Cetus Corporation (Emeryville, California – merged with Chiron in 1991), but was largely developed by other scientists at the corporation, most notably Stephen Scharf and Henry Erlich, and published in 1986 (Mullis et al., 1986; Scharf et al., 1986). The PCR technique was later sold to Roche Molecular Systems for \$300 million in 1991 and has since revolutionised molecular biology.

The starting material for PCR, the 'target sequence', is a gene or segment of DNA. Heating separates the complementary strands of a double-stranded molecule of DNA. Two small pieces of synthetic DNA, each complementing a specific sequence at one end of the target sequence, serve as primers, with each primer binding to its complementary sequence. DNA polymerases (e.g. Taq – thermostable) start at each primer and copy the sequence of that strand. Within a short time, exact replicas of the target sequence have been produced. In subsequent cycles, double-stranded molecules of both the original DNA and the copies are separated; primers bind again to complementary sequences and the polymerase replicates them. In a matter of hours (after many cycles), the target sequence can be amplified many million fold with this amplified genetic information then being available for further analysis.

Reverse transcriptase-PCR (RT-PCR) is used to amplify products of RNA (any kind – mRNA, tRNA, rRNA, etc) to create cDNA, allowing the analysis of gene expression. A cDNA is a single-stranded copy of an RNA sequence and synthesized by the retroviral enzyme reverse transcriptase (RT). This reverse transcription step is performed prior to PCR.

PCR was applied to amplify DNA encoding for CCR4. The cDNA was generated by reverse transcription from mRNA isolated from cells and PCR enabled visualisation of DNA segment bands in UV illuminated ethidium bromide gels. With this qualitative process, the extent of constitutive transcription of CCR4 was assessed in a range of cell types.

Sample mRNA Extraction

Sample mRNA was extracted from CHOK1hCCR4, parental CHOK1, HUT-78, CEM, MOLT-4, EL-4, and T-lymphoblast cells. Initially, total cellular RNA was isolated from the cells using RNeasy B according to the manufacturers instructions. 1×10^6 cells were centrifuged at 13000 rpm for ~ 5 sec and the supernatant was discarded and the cells were homogenized in 400 μ l of RNeasyTM B (Biotex, USA). 100 μ l of chloroform per 1 ml of homogenate was then added, the samples vortexed vigorously for 15 seconds and then cooled on ice for 15 minutes. The samples were then centrifuged at 14000 rpm for 15 minutes, 4 °C. This leads to the formation of two phases: a lower blue phenol-chloroform phase and the RNA-containing upper aqueous phase. The aqueous phase was transferred to a clean eppendorff tube and an equal volume of isopropanol was added. The samples were cooled on ice for 15 minutes and centrifuged again at 14000 rpm for 15 minutes at 4 °C. RNA will then form a precipitate at the bottom of the tube. The supernatants were removed and the RNA pellet washed in 1ml 70% ethanol. The pellets were then dried and re-dissolved in nuclease-free water (Promega, Southampton, UK).

RNA was quantified using a deuterium lamp spectrophotometer, Gene Quant II RNA/DNA calculator (Pharmacia, UK). RNA concentration is measured by the absorbance of 1 μ l of RNA sample diluted in 500 μ l of water at 260 nm. The amount of RNA (in μ g) present in each sample was calculated by the following formula:

$A_{260} \times \text{dilution factor (500)} \times 40^* \times \text{volume of remaining RNA solution in ml (0.048)}$

*assumption is that a solution of 40 μ g/ml ssRNA (50 μ g/ml for dsDNA) has an absorbance of 1 at 260 nm.

ODs were also read at 280 nm and 230 nm to assess the purity of RNA. A value of less than 2 for the OD₂₆₀:OD₂₈₀ ratio indicated protein contamination. A low OD₂₆₀:OD₂₈₀ ratio indicated guanidine contamination. A ratio between 1.8 and 2.0 was considered acceptable.

1 µg of total RNA was dissolved in 8 µl of nuclease-free water and was treated with 1 µl DNase I for possible DNA contamination. DNase I was then inactivated by the addition of 1 µl EDTA and heated at 65° for 10 minutes.

Reverse Transcription (RT) Step

The resulting solution was then mixed with 2 µl oligo(dT) and denatured at 70 °C for 10 minutes. The reverse transcription mixtures were made up in 0.2 ml PCR tubes and contained 4 µl buffer (supplied with enzyme (Invitrogen)), 1µl DTT, 1 µl DNTP's, 1 µl RNAsin and 1µl of reverse transcriptase per sample and, together with the 12 µl mRNA sample, gave a final volume of 20 µl per PCR tube. The final concentration in 20 µl of the constituents was: 1 µM of oligo(dT)₁₂₋₁₈, 0.5 mM from each of the deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP, 10 U/µl Superscript II RNase H⁻Reverse Transcriptase, and 1 U/µl RNAsin (non-competitive ribonuclease inhibitor).

The tubes were placed in a Perkin Elmer Gene Amp 2400 thermocycler (Warrington, UK) and followed a reverse transcription program of: 42 °C for 60 min, 94 °C for 2 min and 4 °C thereafter. The RT products were either used immediately, or briefly stored at -80° C.

PCR Step

A forward and reverse primer was obtained for the CCR4 gene of interest (using Primer 3 Design software available on the internet) from Dr. Nicola Jordan (University of Bath). The DNA sequence that was amplified by PCR was selected in a way that the primers were spanning at least one intron, which would result in the generation of a larger intron-containing PCR product in the case of DNA contamination. Each RT template from an experimental sample, apart from the genes of interest, were also tested for a positive control, the house keeping gene β-actin and a negative control of original RNA before the RT step, to check for DNA contamination. Each PCR reaction was carried out in 0.2 ml

thin PCR tubes in 25 µl total volume containing 0.05 µg cDNA template, 15.9 µl of nuclease-free water and the following final concentrations of constituents (in appropriate volumes to give a final volume of 25 µl per tube): 200 µM of each of the 4 deoxynucleoside triphosphates dATP, dCTP, dGTP and dTTP; 500nM of the forward (sense) primer; 500 nM of the reverse (antisense) primer; 1.25 Unit of ExpandTM High Fidelity enzyme mix, which comprised of 2 DNA polymerase enzymes: Taq and a proofreading polymerase Pwo; and 1 x ExpandTM PCR buffer with Mg²⁺. The polymerase enzymes and PCR buffers were used according to 'ExpandTM High Fidelity PCR System' manufacturer's specifications, and the thermocycler was given the following programme: 15 sec at 95 °C, 15 sec at 57 °C, 15 sec at 72 °C repeated for 30 cycles and two holds, one at the beginning of the programme for 60 sec/95 °C and one at the end for 6 min/72 °C, prior to cooling to 4 °C thereafter. The temperatures set were optimal for the phases of the PCR cycle: 95° C for denaturation of the template, 57 °C for annealing of the chemokine receptor oligonucleotide primers to the open DNA strand and 72 °C for optimum DNA synthesis by the heat stable polymerase enzymes. Dr. N. Jordan optimised cycle composition and number of cycles for PCR amplification of chemokine receptor primers in-house. The PCR products were either loaded immediately on an agarose gel or briefly stored at 4° C.

Human Gene	Product Length (bp)	Primer Sequence
CCR4	349	sense: 5' GAAGAAGAACAAGGCGGTGAAGAT 3' antisense: 5' ATGGTGGACTGCGTGTAAGATGAG 3'

Detection of PCR Products

A 2% agarose gel was made in TBE (10 mM Tris base, 10 mM Boric acid, 2 mM EDTA, pH 8.0), boiled and cooled with the addition of 1 µg/ml ethidium bromide. 5 µl from each tube containing the PCR end products were coloured with 5 µl of Blue Juice (15% (w/v) Ficoll 400, 0.25% (w/v) bromo-phenol blue in water). A 100 base pair ladder comprising of 15 blunt end fragments (100 – 1500 base pair scale) was also coloured with Blue Juice. The samples and ladder were loaded onto the agarose-ethidium bromide gel and run by gel

electrophoresis with 100 V/0.1 mA current (BioRad Instruments). The resulting bands were visualised with a UV illuminator and photographed with a Polaroid camera.

Flow Cytometry (FACS) Analysis

Cells were washed twice in PBS and aliquoted into polypropylene FACS tubes at 1×10^6 cells/tube and resuspended in 95 μ l PBS/20% FBS. The cells received either 1 μ g of PE-conjugated anti-CCR4 mAb (5 μ l), or corresponding PE-conjugated IgG₁, κ isotype control (BD PharMingen), or 5 μ l PBS/20 %FBS negative (vehicle) control and were incubated for 30 minutes on ice, in the dark and with regular mixing. Cells were then subsequently washed twice and resuspended in 500 μ l PBS (or 500 μ l 4 % paraformaldehyde, wrapped in foil and placed in the fridge for future analysis) and immediately analysed using a FACS Vantage (BD Biosciences, San Jose, CA); excitation λ 488 nm, emission λ 578 nm (band pass filter 575/26).

Expansion of Primary Human T Lymphocytes (T Lymphoblasts) in vitro

Bottles of wash media (RPMI 1640) and complete media (RPMI 1640 + 10 % FBS + 100 units/ml penicillin, 100 μ g/ml streptomycin) were warmed to 37 °C in a water bath, and 15 ml of Lymphoprep was aliquoted into 50 ml transparent conical tubes and allowed to reach room temperature in the comparative dark. Peripheral blood was then collected from healthy human donors (free of medication, including paracetamol, NSAIDs, etc) in sterile heparinised syringes (500 U/ml stock: 2 μ l/ml blood). The blood was then diluted 1:1 with pre-warmed wash media in a large (175 cm²) tissue culture flask, mixed and, very carefully, 35 ml of the diluted blood layered on to the Lymphoprep present in the conical tubes (without mixing the two). The conical tubes were then spun at 1500 rpm for 30 min at rtp, with the brake off, isolating the peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation (fig. 2.2).

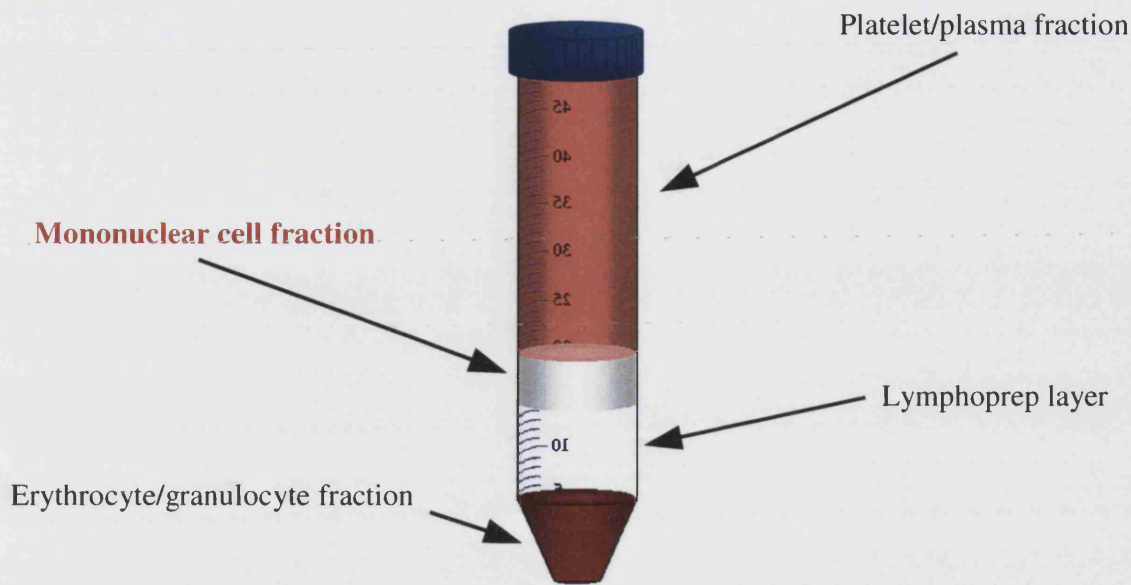


Figure 2.2. Schematic diagram of PBMC separation with Lymphoprep after centrifugation. The diagram is representative of the separated layers observed after centrifugation with Lymphoprep, along with the colour each layer appears, in an undisturbed, transparent conical tube.

After centrifugation, the milky layer containing the mononuclear cells (i.e. lymphocytes and monocytes) was carefully removed with a 3 ml long-necked pastette, being carefully not to aspirate off any of the Lymphoprep, into a new 50 ml conical tube. The remaining volume of the conical tube was filled with wash media and the tube spun at 1500 rpm for 5 min, rtp. The supernatant was aspirated off and the cells were washed a further three times with wash media to ensure that any Lymphoprep present was removed. Following the three washes, the pellet containing PBMCs was resuspended in complete media to the original blood volume (approximately 1×10^6 cells/ml) and transferred to a large (175 cm²) tissue culture flask. The PBMCs were then stimulated (T cells activated to become T lymphoblasts) with staphylococcal enterotoxin B (SEB, 1 µg/ml) for 72 hours, and cultured in a humidified incubator at 37 °C, 5 % CO₂. After three days, the cells were washed three times to remove the SEB and resuspended in fresh complete media to original volume and growth maintained by supplementing every two/three days with IL-2 (48 UI/ml) for four

additions. For experiments (day 12 –13 post-isolation), cells were deprived of IL-2 for two days and allowed to accumulate in the G₀/G₁ stage of the cell cycle prior to use. Cells were resuspended in RPMI 1640 with 0.1 % BSA for 1 hour prior to use in experiments (unless stated otherwise).

In Vitro Generation of Human T Helper 2 (Th2) Cells

Blood samples were collected from healthy adult volunteers and deposited in to 50 ml conical tubes containing 1 ml of anti-coagulant (20 mg/ml EDTA in PBS) per 10 ml of blood. Blood was diluted 1:1 with pre-warmed RPMI 1640 and 35 ml of diluted blood carefully layered on to 15 ml of Ficoll-paque (1.077), and blood separated by density gradient centrifugation, as described above. The PBMCs were removed from the gradient as previously described, and cells washed 3 x (1600 rpm, 5 min, 12 °C) with MACS buffer (PBS + 0.5 % BSA + 2 mM EDTA, pH 7.2, at 4 °C) and cells counted. Cells were resuspended in 80 µl MACS buffer/10⁷ cells, and naïve CD4⁺ T cells isolated using magnetic cell separation. This involved magnetically labelling cells with CD4⁺ MicroBeads (20 µl/10⁷ cells), incubating the cells + beads in the fridge for 15 min, washing 2 x with MACS buffer (1600 rpm, 5 min, 12 °C), resuspending the cells in 2 ml MACS buffer, and separating (positive selection) on a column that is placed in the magnetic field of an AutoMACS separator (Beads and machine from Miltenyi Biotec, Germany). The collected CD4⁺ fraction of cells were then incubated with 20 µl/ml Release Agent (supplied with kit) and incubated in the fridge for 10 min. Subsequently, cells were washed (2 x), counted, resuspended in MACS buffer at 50 µl/10⁷ cells, and 30 µl/10⁷ cells Stop Reagent (supplied with kit) added. A similar process was then repeated for the positive selection of CD45RA⁺ cells within the CD4⁺ cell fraction. Specifically, CD45RA⁺ MicroBeads (20 µl/10⁷ cells) were added to the CD4⁺ fraction of cells and incubated in the fridge for 15 min, followed by washing (2 x) and resuspension in 500 µl MACS buffer. Cells were once again separated using the AutoMACS machine, with the positive fraction collected (i.e. CD4⁺CD45RA⁺ cells), washed 2 x in pre-warmed RPMI 1640 and counted. Isolated CD4⁺CD45RA⁺ cells were cultured at 10⁶ cells/ml in humidified incubators (5 % CO₂ at 37 °C)

in RPMI 1640 medium, supplemented with 10% (v/v) FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% MEM vitamins, 10 µg/ml insulin, 5.5 µg/ml transferrin, 6.7 ng/ml selenium, 50 µM 2-mercaptoethanol, 20 U/ml rhIL-2. Cells were polarised to a Th2-like phenotype by the addition of rhIL-4 (200 ng/ml) and neutralising anti-IL-12 (1 µg/ml) and anti-IFN γ (1 µg/ml) Abs (R & D Systems). Activation of cells was initiated by culturing (2 ml cells/well) in anti-CD3 (1 µg/ml) coated 12 well plates with the above-mentioned media for the first 5 days. Cells were then washed and again cultured in the above media, except with 50 U/ml rhIL-2 (Chemicon, Chandlers Ford, U.K.), in 175 cm² tissue culture flasks. Cells were used between weeks 3 and 4 post-isolation.

Cell Stimulations and Lysis

Generating 'Whole Cell Lysates' (WCL)

Cells were washed, counted and resuspended in appropriate media (e.g. RPMI 1640 or DMEM) for the cell type, supplemented with 0.1 % BSA. For cell numbers refer to the figure legends as this varies depending on cell type and antibody being utilised (but not normally more than 5 x 10⁶ cells/ml). 1 ml aliquots of cell suspension were added to 1.5 ml microcentrifuge tubes and serum starved for 1 h in a 37 °C water bath. If used, cells were pre-incubated with inhibitors or vehicle (commonly DMSO or MQH₂O) for stated time, and then stimulated for indicated times with agonist (e.g. MDC). Reactions were terminated and lysates generated by pelleting cells in a centrifuge (pulse to ~8000 rpm), aspirating off the supernatant, adding 100 µl/10⁶ cells of 1 x sample buffer (see appendix 1 for recipe – samples are thus denatured and reduced), and vortexing for 15 sec. Samples were then placed on ice and then subsequently boiled for 5 min at 100 °C. Samples were stored in a –20 °C freezer until required.

Generating Samples for Immunoprecipitation (IP)

As for WCL generation, cells were washed, counted and resuspended in the appropriate media for the cell type (supplemented with 0.1 % BSA). Cell numbers were at least 1 x 10⁷

cells/ml, but refer to figure legends for exact numbers. 1 ml aliquots of cell suspension were added to 1.5 ml microcentrifuge tubes and serum starved for 1 h in a 37 °C water bath. If utilised, cells were pre-incubated with inhibitors or vehicle for stated time, and then stimulated for indicated times with the appropriate agonist. Reactions were terminated and lysates generated by pelleting cells in a centrifuge (pulse to ~8000 rpm), aspirating off the supernatant, and adding 500 µl ice-cold lysis buffer (see appendix 1). Samples were vortexed for ~15 sec and placed on ice until next step. Cells were subsequently lysed on a rotator at 4 °C for 20 min. Non-soluble material (cell debris) was removed by spinning samples in a microfuge for 10 min at 14000 rpm and 4 °C. Supernatants were transferred to clean 1.5 ml microcentrifuge tubes.

Immunoprecipitation

Cell lysates (generated by the above method) were pre-absorbed with 20 µl of a 50:50 slurry of protein-A or protein-G sepharose (depending on the species source for the antibody being used – see appendix 4) for 30 min on a rotator at 4 °C. Following rotation, samples were spun at 14000 rpm (a quick pulse) to pellet the sepharose beads, and the supernatant removed and transferred to clean 1.5 ml microcentrifuge tubes. If required, 20 µl of the lysates were removed to tubes containing 20 µl 2 x sample buffer and boiled for 5 min (to generate whole cell lysates for control blots) and stored at –20 °C. The remaining lysate volume was then incubated with 2 – 5 µg antibody on a rotator at 4 °C for 2 hr. After 2 hr, 20 µl of a 50:50 slurry of pre-washed protein-A or protein-G sepharose beads were added to the lysate-antibody mixture and incubated for a further 1 hr on a rotator at 4 °C. For immunoblotting, the lysate-bead-antibody mixture was washed 3 x with ice-cold lysis buffer (14000rpm, 4 °C, ~5 sec). The final wash was removed as completely as possible (using a Gilson pipette fitted with a 200 µl gel-loading tip) and the pellet resuspended in 20 µl 2 x sample buffer (denaturing and reducing), vortexed for ~15 sec, and boiled (100 °C) for 5 min. Samples were then stored at –20 °C for future use or utilised immediately.

SDS-PAGE & Western Blotting (Immunoblotting)

Whole cell lysates or immunoprecipitates were essentially resolved by processes first described by Ornstein, Davis, and Laemmli (DAVIS, 1964; Laemmli, 1970; ORNSTEIN, 1964). Briefly, electrophoresis was carried out using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) method. The excess presence of the ionic detergent (SDS) and thiol reagent (i.e. 2-mercaptoethanol – a reducing agent) in the sample buffer, along with the process of boiling the samples at 100 °C, has the effect of denaturing the proteins by SDS wrapping around the polypeptide backbone and the thiol reagent cleaving the disulphide bonds. This results in the proteins being fully dissociated into their individual polypeptide subunits. Most polypeptides will bind SDS in a constant weight ratio (1.4 g SDS:1 g of polypeptide) in the conditions mentioned above. This leads to the negation of the effect of the intrinsic charges of the polypeptides due to the negative charges provided by the bound SDS, and thus the SDS-polypeptide complexes all essentially have the same negative charge and shape and, consequently, will migrate through the gel according to polypeptide size only. A discontinuous buffer system was employed, which consisted of a large pore non-restrictive (stacking) gel overlaying a smaller pore resolving gel. The principal of separation follows that the pH 6.8 of the stacking gel and sample buffer results in the presence of weakly ionised glycine and thus its mobility is low. The chloride ions in the running buffer are completely ionised and therefore have higher mobility, with the mobility of the proteins being at an intermediate between the two different ion types. The application of a voltage causes the leading chloride ions to migrate away from the trailing glycine ions setting up a zone of lower conductivity, higher voltage gradient and higher pH behind the chloride ions. This zone accelerates the glycine to keep it up with the chloride ions and this boundary moves through both stacking and resolving gels, with chloride ions overtaking any proteins in front. The proteins have a higher velocity than glycine meaning that the proteins are concentrated into thin zones (stacks) in order of decreasing mobility. When the proteins enter the higher percentage resolving gel their mobilities are reduced so that they migrate slower than the glycine ions allowing the proteins to escape the 'stack'. The increase in pH of the resolving gel (pH 8.8) accelerates the trailing ions (glycine) by increasing its net negative charge and thus mobility increases. This leads to the glycine ions overtaking some of the proteins and migrate directly behind the chloride ions allowing the proteins to

resolve. Regulation of the pore size of the gel is obtained by the effective use of differing concentrations of acrylamide (higher the concentration the smaller the pore size – the stacking gel generally has a pore size larger than the resolving gel), and in the presence of polymerising compounds (APS and TEMED – TEMED is a tertiary amine base that catalyses the formation of oxygen free radicals from APS causing bis-acrylamide (or acrylamide) to polymerise) leads to the generation of polymerised acrylamide monomers and crosslinked of the chains. The size of the pore size required will depend upon the size of the polypeptide of interest.

SDS-PAGE

Whole cell lysate or immunoprecipitates were separated via minigel SDS-PAGE using BioRad Mini Protean III equipment (Bio-Rad Laboratories, Hemel Hempstead, UK). All gels were 1 mm thick and stacking gels were always 5 % bis-acrylamide. Bis-acrylamide percentage for resolving gels varied between the differing proteins of interest (see appendix 1 & 5 for gel recipes and bis-acrylamide gel percentages utilised for the varying proteins of interest). The glass plates were set-up according to manufacturer's instructions and the resolving gel cast (~ 4.5 ml of Resolving Buffer/gel), overlaid very carefully with MQH₂O to ensure a smooth and flat gel, and left to polymerise for ~ 30 min. After polymerisation, the water was completely removed and Stacking Buffer (with APS and TEMED added immediately prior to casting) poured on top of the resolving gel to the top of the small glass plate with a comb (containing the appropriate number of teeth (10 or 15)) put in place and the gel allowed to set (~ 15 min). Subsequent to stacking gel polymerisation the comb was removed and the wells washed 3 x with MQH₂O and then filled with Running Buffer (see appendix 1 for recipe) and samples added to appropriate wells with a broad molecular weight range marker included (Bio-Rad) for ease of protein band identification. Minigels were electrophoresed at 70 V until the samples had passed the stacking gel and then the voltage increased to 170 V for the remainder of the gel.

Gel Transfer

The proteins from the electrophoresed minigels were transferred onto nitrocellulose membranes by the semi-dry transfer method. The nitrocellulose membrane was placed on top of 4 pieces of 3 MM filter paper cut to the same size as the gel (8.5 cm x 5.5 cm), all of which were soaked in Semi-Dry Transfer Buffer (see appendix 1 for recipe) and placed on the anode of the transfer apparatus. The stacking gel was cut away from the resolving gel and the resolving gel rinsed in Semi-Dry Transfer Buffer and carefully placed on to the nitrocellulose membrane. 4 more layers of pre-soaked (in transfer buffer) filter paper were placed on top of the gel to form a 'sandwich'. All layers of the 'sandwich' were rolled to remove all air bubbles. The cathode was plate was applied to the top of the 'sandwich' and proteins were transferred for 1 hr at 0.8 mA per cm² of gel.

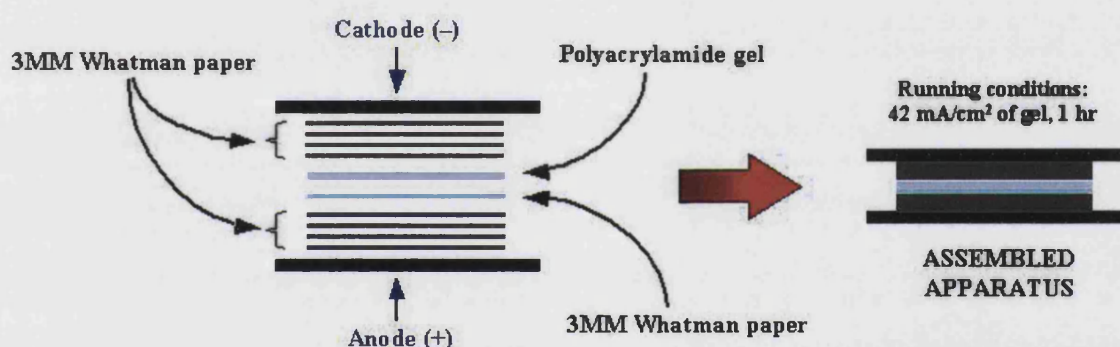


Figure 2.3. Construction of a single gel membrane sandwich, with running conditions.

Western Blotting (Immunoblotting/Immunostaining)

Following gel transfer, membranes were washed (2 x) in tris-buffered saline (TBS) and protein bands stained with Ponceau S solution (seconds) as an indication of efficient and equal transfer of protein bands across the membrane. Ponceau S was removed by washing the membranes (1 x) with Semi-Dry Transfer Buffer until the membranes were no longer stained. Membranes were then briefly rinsed (3 x) with TBS and blocked for the appropriate length of time with either non-fat dry milk powder in TBS or BSA in TBS (for

blocking conditions of the various antibodies refer to appendix 5) at rtp on a rocking platform. After blocking, the membranes were briefly rinsed 3 x in TBS and then membranes incubated with primary antibody (once again, refer to appendix 5 for conditions) at rtp on a rocking platform. After appropriate incubation time with primary antibody, the membranes were washed 5 x 5 min with TBS + 0.1 % Tween-20 (TBST). Suitable secondary antibody (conjugated to horse radish peroxidase (HRP)) diluted in TBST was then applied to the washed membranes for 1 hr at rtp (with rocking) before membranes were washed 3 x with TBST and 2 x with TBS. Protein bands were visualised with Amersham Enhanced Chemiluminescence (ECL) reagent (incubation of 1 min/membrane) according to manufacturer's instructions and membranes wrapped in cling film and exposed to autoradiography film.

Stripping of Western Membranes

Membranes to be reprobed with primary and secondary antibody (commonly for verification of equal loading) were washed 3 x with TBST and submerged in pre-warmed (60 °C) Stripping Buffer (refer to appendix 1 for recipe) for 30 min at 60 °C. Following rigorous washing of membranes with TBST, membranes were blocked and reprobed with appropriate primary and secondary antibodies, as previously described in 'Western Blotting'.

In Vitro Lipid Kinase Assay

Cells were washed, stimulated and immunoprecipitated as previously described in the 'Immunoprecipitation' section, with PI3K lysis buffer used instead of 'normal' lysis buffer (see appendix 1 for recipes). More specifically, the samples were immunoprecipitated using an antibody against the catalytic subunit of the PI 3-kinase of interest (i.e. p110 γ , p110 δ , C2 α , C2 β) or p85 (to determine the activity of all class I PI3K isoforms) for 2 h, and rotated at 4°C. Samples were then incubated for a further hour with 40 μ l 50:50 protein A slurry. Following protein A incubation period, immunoprecipitates were washed 3 x in Buffer A, 3 x in Buffer B, and finally 2 x with Tris-NaCl-EDTA (TNE) buffer (see

appendix 1 for recipes). The first two buffer washes were carried out by pelleting the beads at 14000 rpm for 30 sec, the third group of buffer (i.e. TNE) washes were carried out by pelleting the beads at 14000 rpm for 2 min (all washes were carried out at 4 °C). After removal of the final wash as completely as possible (by using a Gilson pipette fitted with a 200 µl gel-loading tip), samples were resuspended in 50 µl TNE, 10 µl (20 µg) phosphatidylinositol (PI) and 10 µl 100 mM MgCl₂. Note that the PI3K inhibitors, LY294002 and wortmannin, were incubated with the immunoprecipitates at this point for 30 mins and 15 mins, respectively, with dilutions of inhibitor in TNE (the PI and MgCl₂ were added after the incubation period with the PI3K inhibitors). Reactions were started by the addition of 5 µl ATP Reaction Buffer (see appendix 1) and allowed to proceed for 10 min at 25°C, with continuous agitation. Reactions were terminated by adding 20 µl 5N HCl and 160 µl chloroform/methanol (1:1) and phases separated by centrifugation at 14,000rpm for 10 min at rtp. 50 µl of the lower phase was removed and spotted onto 1% oxalate-treated thin-layer chromatography (TLC) plates (in 2 x 25 µl loading steps) and resolved by TLC in chloroform/methanol/water/ammonium hydroxide (60:47:11.3:2). Laned silica gel TLC plates were pre-treated by submerging the plate in 1 % potassium oxalate for a few seconds and dried in an oven overnight at 100 °C, before being removed and allowed to reach rtp before use (or storage). The solvent tank was pre-equilibrated for at least 3 hr prior to the addition of the plate. The plate was removed from the tank when the solvent front had reached within 1 cm of the top of the plate. [³²P]-labelled lipids were visualised by exposure to autoradiography film at -80°C.

Densitometry

Densitometry was performed on IVLK films to provide a more qualitative form of result. This method was performed with the use of the Gene Snap 4 and Gene Tools 3.00.22 programs (SynGene, Cambridge, U.K.). Gene Snap 4 was utilised to take an electronic picture of the film (following the program instructions) and the picture imported into Gene Tools to perform the density analysis of each band and with automatic background deduction incorporated. The generated values were subsequently exported to Microsoft

Excel 2000 program (Microsoft Corporation, Redmond, WA, U.S.A.) for graphical conversion.

Measurement of D3 Phosphoinositide Lipids

Phospholipid Labelling and Extraction

Cells were washed 3 x with pre-warmed phosphate-free DMEM supplemented with 20 mM HEPES and 0.375 % sodium bicarbonate (pH 7.2), incubating cells for 10 min at 37°C in between washes to deplete cells of phosphate (cells spun at 1600 rpm, 5 min, rtp). Following the final wash, cells were resuspended at 2×10^7 cells/ml in phosphate-free DMEM + 5% phosphate-free FBS and labelled with 1mCi [32 P] orthophosphate/ 1×10^8 cells (i.e. 200 μ Ci/ml), for 4 h at 37°C. Dialysing against saline for 18 hr attained phosphate-free FBS, which was then filter sterilised, aliquoted and stored at -20 °C. Dialysis tubing was prepared by boiling twice for 5 min in 5 mM EDTA + 200 mM Na₂CO₃. Proceeding the 4 hr incubation, 32 P-labelled CEM cells were then washed 3x in pre-warmed phosphate-free DMEM and resuspended at 2×10^7 cells/108 μ l in DMEM (or 97.2 μ l DMEM if PI3K inhibitors are used) in 1.5 ml screw-capped Sarstedt tubes. Cells were stimulated, as indicated in figure legends, by the addition of 12 μ l of 10x agonist (or incubated with LY294002 or wortmannin (10.8 μ l addition of 10x inhibitor) for 30 and 15 min, respectively, beforehand), at 37 °C. Reactions were quenched by the addition of ice-cold 500 μ l chloroform/methanol/H₂O (32.6:65.3:2.1 % v:v:v) and vortexing for ~15 sec to produce a primary extraction phase. Phases were separated by the addition of 200 μ l freshly prepared chloroform containing 10 μ g/ml Folch lipids, and 200 μ l of 5 mM tetrabutylammonium sulphate (TBAS – see appendix 1 for recipe), then vortexed for ~30 sec and centrifuged at 8000 rpm (~1000 g). The lower phase was carefully removed (using a Gilson pipette attached with a 200 μ l gel loading tip) to clean 1.5 ml Sarstedt tubes containing 400 μ l 0.1 M HCl + 5 mM EDTA, with samples vortexed and centrifuged at 8000 rpm for a further 5 min to separate phases. The lower phase was removed and transferred to clean 1.5 ml Sarstedt tubes and dried *in vacuo* (medium heat - ~1 hr). The

dried lipids were then deacylated by the addition of 1 ml 25 % (w/v) aqueous methylamine/methanol/N-butanol (4:4:1 v/v/v) and incubated in a 53 °C water bath for 40 min, and then rapidly cooled on ice for a further 5 min before samples dried *in vacuo* overnight (medium heat). The deacylation generates glycerophosphatidylinositol (GroPI) derivatives that are soluble in water. Thus, 500 µl MQH₂O was added to the dried samples followed by 600 µl N-butanol/petroleum ether (bp 40 – 60 °C)/ethyl formate (20:4:1 v/v/v). After vigorous vortexing, samples were centrifuged at 8000 rpm for 5 min to separate phases, and the upper layer removed and discarded. The aqueous phase was washed by adding a further 600 µl of the above-mentioned N-butanol/petroleum ether/ethyl formate solution, centrifuged at 8000 rpm for 5 min, with the upper organic phase discarded as before. The lower aqueous phase was dried *in vacuo* overnight (medium heat) and samples then stored at –80 °C until analysed by high performance liquid chromatography (HPLC) (Ward, 2000).

High Performance Liquid Chromatography (HPLC) Analysis

The deacylated samples were analysed by anion-exchange HPLC analysis using a 12.5 cm Partisphere SAX column (Whatman, Maidstone, UK). The dried lipids were redissolved in 90 µl MQH₂O by vortexing (and by the use of a sonicating water bath), with non-soluble material sedimented at 14000 rpm for 1 min. The sample was automatically applied with a Jasco Intelligent sampler 851-AS to an anion exchange Whatman Partisphere SAX column at a flow rate of 1 ml/min. The lipids were eluted from the column using a gradient based on Buffer A (MQH₂O) and Buffer B (1.25 M ammonium phosphate [(NH₄)₂HPO₄], pH 3.8 (adjusted with HPO₄ at 25 °C)), with both buffers filtered and degassed (with bubbling nitrogen for 30 min) prior to use. The mobile phase gradient was fed into the column by a Jasco Intelligent HPLC pump PU-980 connected to a Ternary gradient unit LG-980-02 and was as follows: 0 min, 0 % Buffer B (B); 5 min, 0 % B; 45 min, 12 % B; 60 min, 30 % B; 61 min, 100 % B; 65 min, 100 % B; 66 min, 0 % B; 90 min, 0 % Buffer A and B. The eluate was fed into a Canberra Packard A-500 Flo-One on-line beta-radiodetector where it was mixed with 3 volumes (i.e 3 ml/1 ml eluate) of Flo-Scint IV scintillation fluid. The results were analysed by the Flo-One data program (Radiomatic, USA). Retention times for individual GroPIs have been described elsewhere (fig. 2.4) (Ward, 2000).

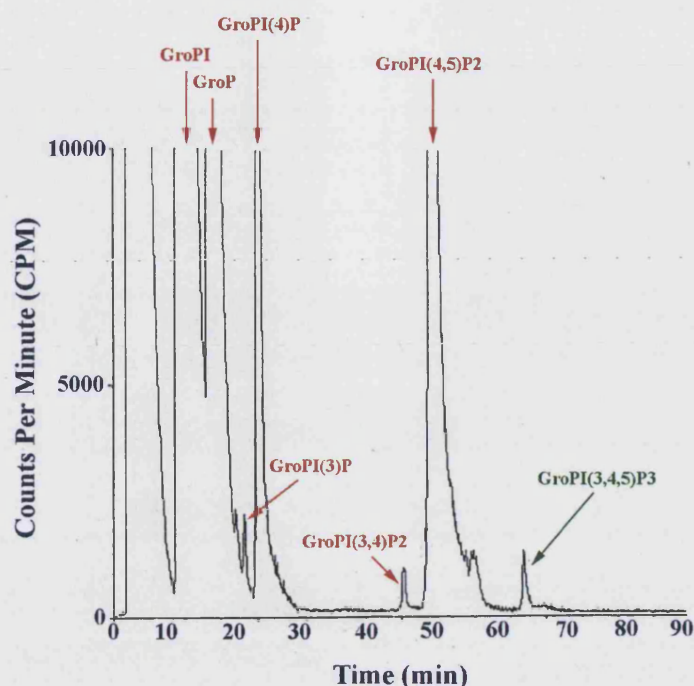


Figure 2.4. HPLC elution profile of deacylated [^{32}P]-labelled phosphoinositides derived from an activated leukaemic T cell line (Ward, 2000).

Chemotaxis Assay – NeuroProbe Reusable MB Series 96-Well Chemotaxis Chambers (CEM Cells)

CEM chemotaxis assays were conducted in 96-well reusable chemotaxis chambers (NeuroProbe, Gaithersburg, MD, U.S.A.). Cells were washed twice and resuspended in RPMI 1640 media + 0.1 % BSA at 1×10^6 cells/ml and serum starved for 1 hr at 37 °C. The chamber was assembled according to manufacturer's instructions (fig. 2.5), with 365 μl of agonist (diluted in RPMI 1640 + 0.1 % BSA) added to the lower wells (avoiding formation of air bubbles) and a polyvinylpyrrolidone-free polycarbonate membrane (5 μm pores) filter was carefully stuck firmly to the plate. The gasket was positioned onto pins on underside of chamber lid and lid gently closed with even pressure across the top of the chamber. 200 μl of cell suspension were added to the upper wells, then covered with parafilm and incubated at 37 °C (5 % CO_2) for 90 minutes. Following incubation, the upper wells were aspirated off and 200 μl of versene was added to each well for 20 minutes

at 4 °C. The versene was then aspirated off and the chamber dismantled. The 96-well plate and attached membrane were spun at 1500 rpm for 10 minutes to pellet cells and the supernatant carefully aspirated off. The cells were resuspended in 100 µl RPMI 1640 + 0.1 % BSA. Cell numbers were determined from standard curves of the same cells using 20 µl Cell Titer 96 AQueous reagent per well with plates incubated at 37 °C for colour changes to develop before the plate is read at 490 nm on a Dynatech MR5000 plate reader (Dynatech Laboratories, Billingshurst, U.K.). Results were presented as a chemotactic index (fold increase over basal) with statistical analysis performed using Student's *t* test.

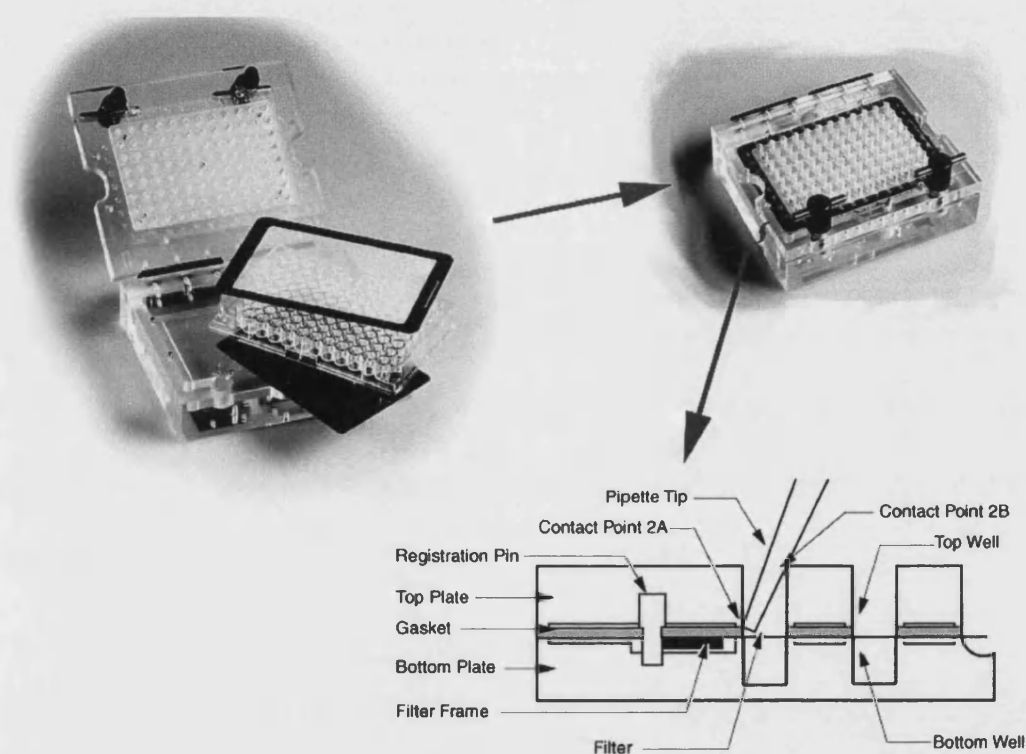


Figure 2.5. The MB Series 96-well reusable chemotaxis chamber and assembly (individual images from www.neuroprobe.com).

Chemotaxis Assay – NeuroProbe Disposable ChemoTx System (Th2 cells)

Th2 cell chemotaxis was conducted with the faster and recently available disposable ChemoTx plates (more consistent/reliable than the chambers used for CEM cell chemotaxis, but were not available at the beginning of those studies) (NeuroProbe, Gaithersburg, MD, U.S.A.). Cells were washed twice and resuspended in RPMI 1640 media (without phenol red) + 0.1 % BSA at 1×10^7 cells/ml. Cells were labelled with 5 μ g/ml calcein-AM (Molecular Probes, Leiden, Netherlands) for 30 minutes at 37 °C on a rocker. Cells were then washed (x 2) and resuspended at 4×10^6 cells/ml in above-mentioned media and starved for 1hr at 37 °C. The bottom wells of the plate were carefully loaded with 29 μ l of agonist, avoiding the formation of air bubbles, and then carefully overlaid with the 5 μ m membrane/filter. 25 μ l (equivalent to 100,000 cells/well) of cell suspension were dispensed on top of the filter for each well and the plate covered with the supplied lid and placed in a humidified incubator at 37 °C for 75 minutes (fig. 2.6). After incubation period, washing away with PBS and scraping the filter top with 3MM chromatography paper removed non-migrated cells. The plate + filter were read on a fluorescent plate reader (Fluoroskan Ascent plate reader, Thermo Labsystems, Finland) using a filter pair of 485 nm (excitation) and 538 nm (emission). Results were presented as a chemotactic index with statistical analysis performed using Student's *t* test.

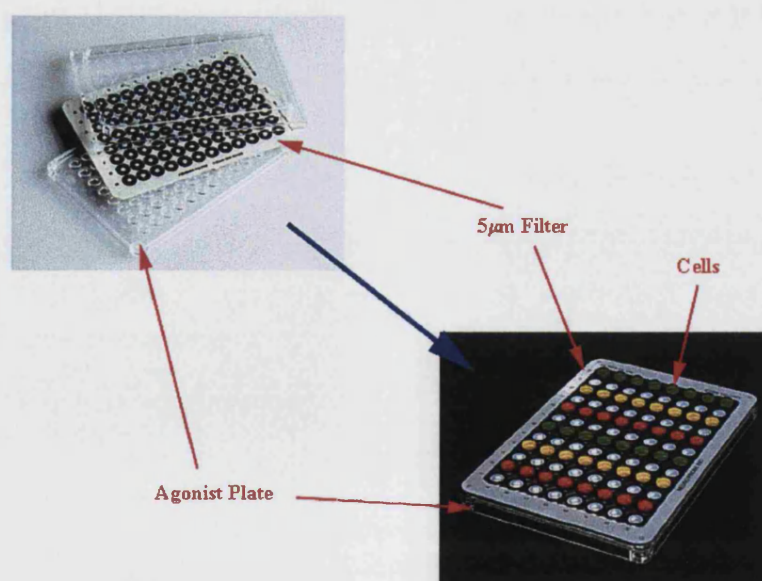


Figure 2.6. The dispensable ChemoTx system and assembly (individual images from www.neuroprobe.com).

Fluorometric Imaging Plate Reader (FLIPR) Assay – Calcium Mobilisation

Agonists were prepared in Assay Buffer (HBSS/20 mM HEPES (without Phenol Red indicator) + 0.1 % BSA w/v) at 3 x required final concentration and aliquoted (> 60 µl) in to a round bottom 96-well plate. Fluo-4 AM (50 µg) was dissolved in 22 µl DMSO followed by the addition of 22 µl of the non-ionic surfactant pluronic F-127 acid (20 % solution in DMSO) to aid solubilisation of the AM ester. Both Fluo-4 and pluronic F-127 supplied by Molecular Probes (Eugene, Oregon, U.S.A.). The Fluo-4 mix was then added to 22 mls of Assay Buffer with Brilliant Black (100 µM final) and probenecid (2.5 mM final) – Loading Buffer (enough for 2 x 96-well plates).

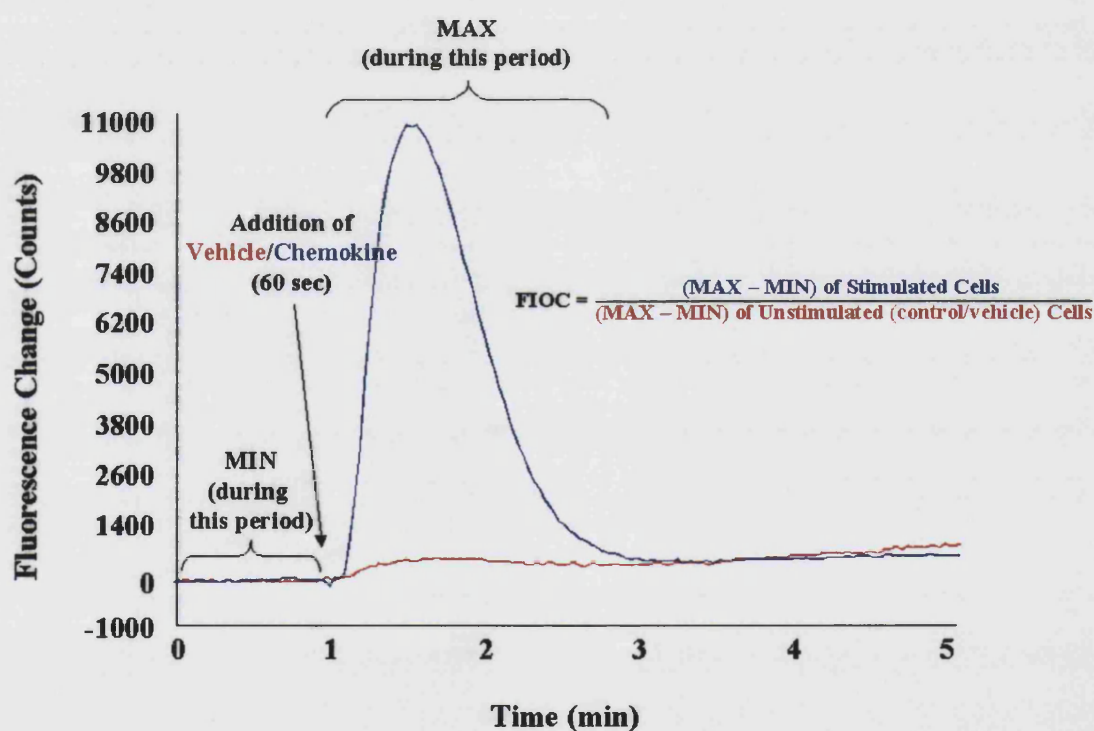


Figure 2.7. Analysing results generated using the FLIPR system. The figure represents a typical result obtained on the FLIPR machine for unstimulated (vehicle-treated) and stimulated cell samples. The fold increase over control (FIOC) was calculated as shown, with the MAX-MIN value derived from the maximum fluorescence counts obtained upon stimulation (vehicle or ligand) divided by the minimum fluorescence counts observed prior to stimulation.

CEM/Th2 cells were washed in Assay Buffer and resuspended at 1×10^6 cells/ml in Loading Buffer. If antagonists are being used, then cells are resuspended in Assay Buffer with inhibitor for required preincubation time (taking into account the subsequent 30 min Fluo-4 incubation time) before the addition of the Fluo-4 mix with probenecid and Brilliant Black (Sigma Aldrich, Gillingham, U.K.). The anion-exchange protein inhibitor probenecid was used to prevent the extrusion of Fluo-4 from the cells by organic ion transporters. 100 μ l of cell suspension (100,000 cells) were added to each well of a black-walled, clear-bottomed, poly-D-lysine coated plate and incubated for 30 min at 37 °C, 5 % CO₂ and 95 % O₂. The presence of Brilliant Black (a quenching agent) in the Loading Buffer negates the need for washing. After loading, the plate was spun at 1200 rpm for 5 min at RT. The cell plate was loaded into the FLIPR (Molecular Devices Ltd, Wokingham, U.K.) and a signal test was taken (also allows the determination of any effect of the inhibitors upon Fluo-4 loading of the cells) and laser power adjusted to obtain a basal level of ~10,000 Fluorescence Intensity Units (FIU). The cells were then excited at 488 nm using the FLIPR laser and fluorescence emission determined used a CCD camera with a bandpass interference filter (510–560 nm). Fluorescence readings were taken at 1 s intervals for 60 s and a further 150 readings were taken at 2 s intervals. Agonist (50 μ l) was added (dispense speed 30 μ l/sec) after 1 min using the FLIPR. Raw fluorescence data was exported for each well and tabulated versus time within Microsoft Excel (Microsoft Corporation, Redmond, WA, U.S.A.). Data was then imported into Origin 6.0 (Microcal Software Inc., Northampton, MA, U.S.A.) and the peak response over basal determined, as a fold increase (fig. 2.7), and curves fitted with the logistic non-linear curve fit equation:

$$y = \frac{A_1 - A_2}{1 + (x / x_0)^p}, \text{ where:}$$

p = power

x₀ = centre (i.e. EC₅₀)

A₁ = initial Y value (i.e. min. response)

A₂ = final Y value (i.e. max. response)

and where $y(x_0) = (A_1 + A_2) / 2$

and $(x_1 / x_0)^p = (p - 1) / (p + 1)$

N.B. the x-axis has to be set to a logarithmic scale.

‘Concorde’ Calcium (MERLIN) Assay

Measurement and visualisation of free intracellular calcium in CHOK1hCCR4 cells was performed using the Concorde (MERLIN) system. Cells were grown on thin (No. 1, 22 mm diameter) coverslips (BDH) until approximately 20% confluent. Cells were then washed (x 2) with media and starved for 1 hour (37 °C) prior to washing (x 3) in physiological salt solution (PSS – see appendix 1). Cells were then loaded with 5 μ M Fura-2-AM (in PSS) for 30 minutes in the dark. Excess Fura-2 was removed by washing the cells three times in PSS and cells left for 15 minutes in the dark. The coverslip was then transferred to an experimental chamber (at 37 °C) mounted on the stage of an inverted microscope (Zeiss Axiovert 100). The bathing medium consisted of 250 μ l PSS. Levels of intracellular-free calcium were then measured using the MERLIN high-speed ratiometric imaging system (MERLIN 2.0, Life Science Resources Ltd, Cambridge, UK) and inverted microscope equipped with an UltraPix FK1000 cooled digital camera and a SpectraMASTER high-speed monochromator (both purchased from Life Science Resources Ltd). Cells were stimulated with MDC or TARC (after approximately 60 sec of basal recording) whilst alternately excited with 340 and 380 nm wavelengths. Fura-2 fluorescence emission intensity was measured at λ 510 nm and the 340/380 nm fluorescence ratios (Rf) were calculated for individual cells and the mean calculated. Results were expressed in a variety of formats and are explained, in detail, in the appropriate figure legends.

N.B. Fura-2 AM is an example of an excitation-shifted or dual excitation indicator and therefore is a dual wavelength probe. Thus, when Fura-2 is not bound to calcium the excitation maximum is 372 nm (but commonly use 380 nm) but when calcium is present the excitation maximum shifts to 340 nm. The emission maximum is always 510 nm. This means that Fura-2 is a useful indicator in ratio-metric calcium assays, such as the ‘Concord’ assay. However, the FLIPR system does not use a ratio-metric system allowing for the use of the calcium indicator Fluo-4. Fluo-4 has the big advantage of a very large fluorescence intensity increase in response to calcium binding allowing for a more sensitive analysis, ideal for the FLIPR system.

Arno-GFP Localisation by Confocal Microscopy

HUT-78 cells were transiently transfected with DIMRIE-C reagent (Invitrogen, Paisley, UK). 0.5 ml of pre-warmed (37 °C) serum-free Optimem medium (Life Technologies, Paisley, UK) was added to each well of a six-well plate followed by the addition of 6 µl DIMRIE-C reagent. In a separate tube, 8 µg/ml plasmid DNA (Arno-GFP (gift from Dr. P. Cullen, University of Bristol, UK)) solution was made-up in serum-free Optimem and 500 µl added to each well of the six-well plate (giving 4 µg/ml DNA final). Wells were mixed gently and the DNA and liposomal reagent were left to complex for 30 min at rtp. HUT-78 cells were washed and resuspended at 2×10^7 cells/ml in serum-free Optimem, and then 200 µl of this cell suspension (4×10^6 cells) was added to each well of six-well plate and mixed. The plate was incubated at 37 °C/5% CO₂ for 4 hours, after which a further 2 ml of Optimem + 15% FBS was added to each well and incubated for a further 20 hours. Coverslips were coated with 3 ml of 5 µg/ml poly-L-lysine solution (in 100 mM borate buffer (sodium borohydride), pH 8.3) for 2 hours at rtp (or overnight at 4 °C), and then washed 2 x in PBS before use. Following the 20 hr incubation, the transfected cells were washed 2 x in RPMI 1640 and serum-starved for 2 hours. 1 ml of cells (4×10^6 cells/ml) were then transferred to a clean six-well plate containing the poly-L-lysine coated coverslips and allowed to adhere for 30 min at 37 °C. Cells were then stimulated for indicated times with 10 nM MDC or vehicle. Following required stimulation time, the media was aspirated off with reactions terminated and cells fixed by the addition of freshly prepared 2 ml of ice-cold 4% paraformaldehyde/1% glutaraldehyde (in PBS) solution for 10 min at rtp. Coverslips were then washed 3 x in PBS and carefully mounted onto a slide with 20 µl Mowiol (Calbiochem, Nottingham, UK) mounting medium (containing the anti-quenching reagent DABCO (Sigma, Gillingham, UK)) and left to dry in the dark overnight, at rtp. Slides were examined on an Olympus inverted stage confocal microscope (Olympus Optical, Hamburg, Germany) using Fluoview software.

3

RESULTS I

3. RESULTS I

3.1 Results I: Role of PI3K in CCR4-Mediated Responses

Introduction

Ligation of chemokine receptors with their respective ligands leads to the activation of various intracellular signalling pathways with resultant induction of a cellular function, such as cell spreading, proliferation, cytokine secretion and cell migration to name a few. One of the most widely studied chemokine-activated signalling cascades is the PI3K-associated pathway, which has been implicated as essential for a cells ability to detect a chemokine gradient and directionally migrate in response to chemokine receptor ligation on a variety of cells (Funamoto et al., 2002; Iijima and Devreotes, 2002; Sadhu et al., 2003b; Sadhu et al., 2003a; Wang et al., 2002b; Weiner et al., 2002). The PI3K family consists of several evolutionary conserved lipid kinases, divided into 3 classes, thought to play a role in a multitude of cellular functions and responses (reviewed in the 'Introduction' and more comprehensively in (Fruman and Cantley, 2002; Vanhaesebroeck et al., 2001)).

There is currently no published data on the activation and role, if any, of PI3K downstream of the activated CCR4 receptor in any cell type. In this chapter, we show that the CCR4 ligands, MDC and TARC, are able to activate the PI3K pathway resulting in the accumulation of PI(3,4,5)P₃ and the phosphorylation of the downstream effector PKB. However, the activation of PI3K isoforms appears to be dispensable for MDC and TARC-mediated migration of T cells.

Validation of Cell Models for Expression of CCR4

It is important to determine the presence and extent of expression of the receptor of interest (in this case CCR4), since receptor presence defines the capacity of the cell to respond to the receptor's ligands (i.e. MDC and TARC). In order to demonstrate that the cells utilised

throughout this thesis express CCR4, PCR and FACS analyses were carried out to establish both message and cell surface CCR4 protein expression, respectively. PCR analysis demonstrated that CCR4 mRNA was present in 5 of the 7 cell types tested, with the CHO parents and the EL-4 mouse T cell leukaemic line lacking CCR4 message (fig. 3.1). Flow cytometry (FACS) analysis revealed that those cells, CHOK1hCCR4, MOLT-4, CEM, HUT-78 and T-lymphoblasts, that tested positive for possessing CCR4 mRNA, express cell surface CCR4 to varying extents (fig. 3.2). CHOK1hCCR4, T-lymphoblasts (T-blasts) and HUT-78 cells exhibit noticeable levels of CCR4 cell surface expression. However, MOLT-4 cells demonstrate minimal quantities of cell surface CCR4. This thesis has principally utilised the CEM cell line and human peripheral blood-derived T cells differentiated *in vitro* toward a Th2 phenotype. CEM cells constitutively exhibit substantial amounts of cell surface CCR4 (fig. 3.2A). Various studies have established the expression of CCR4 on human Th2 cells (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Zhang et al., 2000). Fig. 3.2, B highlights the effectiveness of the method used here to generate CCR4-expressing Th2-like cells, although the amplitude of CCR4 expression is not as great as that observed on CEM cells.

Expression of the PI3K isoforms and the Regulators of their Lipid Products

It is imperative for this results chapter to establish the expression of the PI3K isoforms found in the cell types employed in this work. As expected, the T lymphocyte classes of cells (CEM, Th2, T-lymphoblast, and Jurkat) express each of the isoforms immunoblotted for, namely p110 δ , p110 γ , C2 α and C2 β (fig. 3.3). The relative expression of these isoforms between the cell lines varies, except for p110 γ , which is similar across the cell range. The p110 δ isoform appears to be substantially lower in the two cell lines, CEM and Jurkat, when compared to the 'primary' T cells Th2 and T-lymphoblast (fig. 3.3, *top blot*), even taking into account the variability in protein quantities between the samples. The same samples have been used for all 4 PI3K isoform blots, thus although there was no protein assay performed on the cell lysates to demonstrate similar levels of total protein, blots were stripped and reprobed for β -actin (a cytoskeletal protein that is a widely used housekeeping gene internal control for quantitative RT-PCR reactions, and at the protein

level for Western blot analysis) to ascertain the equality of protein amounts loaded across the lanes. Additionally, the two class II PI3K isoforms, C2 α and C2 β , appear to demonstrate comparable levels of expression across the cell range, but taking into account the substantially higher protein levels loaded for the peripheral blood-derived T cells, then this would suggest that expression is actually significantly lower in Th2 and T-lymphoblast cells. Unsurprisingly, CHOK1hCCR4 cells lack the leukocyte-restricted PI3K isoforms p110 γ and p110 δ , along with PI3K-C2 β . The two lipid phosphatases PTEN and SHIP regulate the levels of D3 phosphoinositide lipids, and therefore play a pivotal role in the PI3K pathway. Jurkats lack both phosphatases SHIP and PTEN, whilst CEMs lack just PTEN at the protein level, as previously described by Freeburn et al. (Freeburn et al., 2002). The 'normal' T cells (T-lymphoblast and Th2) express both lipid phosphatases, with CHOK1hCCR4 cells lacking the 5-inositolphosphatase SHIP (fig. 3.3, *bottom two blots*).

Figure 3.1. PCR analysis of CCR4 mRNA expression in various cell types.

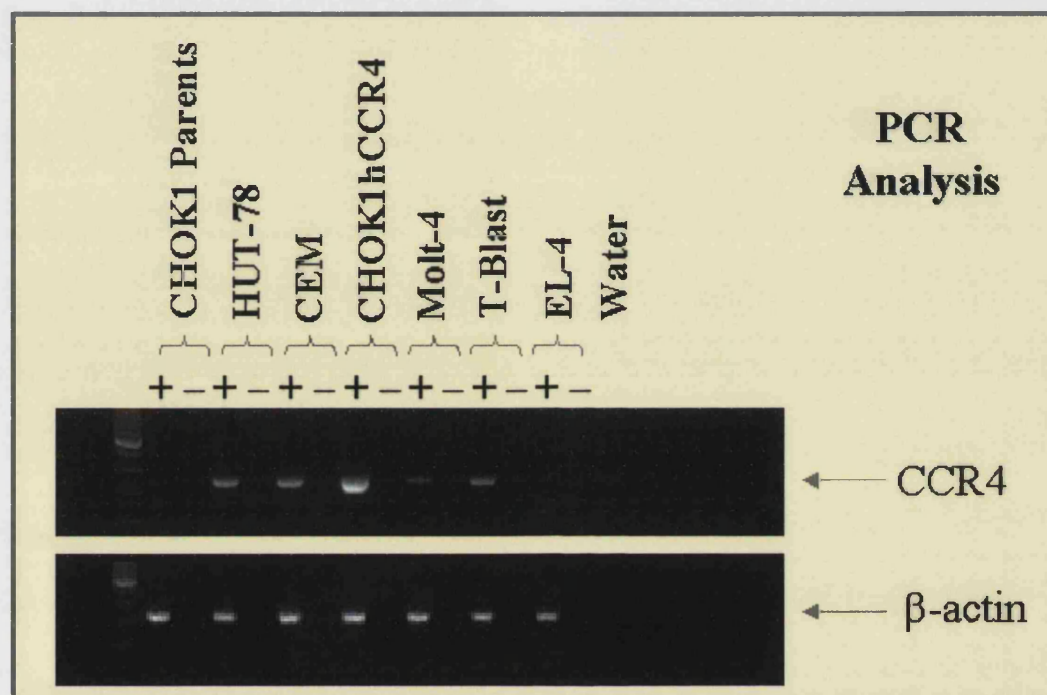


Figure 3.1. PCR analysis of CCR4 mRNA expression in various cell types. PCR was performed on mRNA extracted from each of the cell types. All cells demonstrate the presence of CCR4 mRNA except CHOK1 parents and EL-4 cells. β -actin band demonstrates the generation of cDNA in the positive samples. Result of 1 experiment.

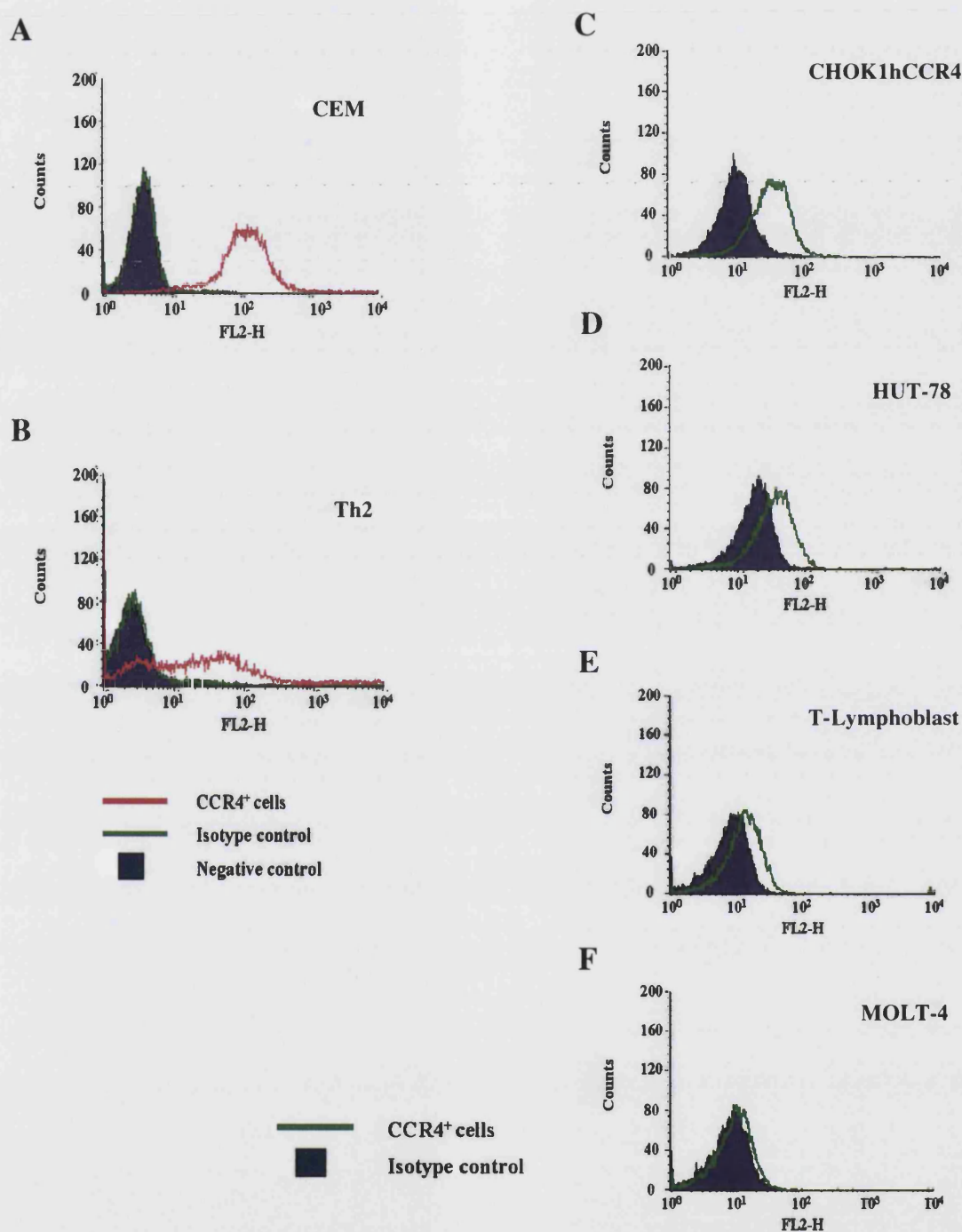
Figure 3.2. Expression of cell surface CCR4 on various cell types.

Figure 3.2. Expression of cell surface CCR4 on various cell types. Expression of CCR4 was assessed by immunostaining 1×10^6 cells $1 \mu\text{g}$ of R-phycoerythrin-conjugated mouse anti-human CCR4 mAb (1G1), or PE-conjugated IgG $_{1, \kappa}$ isotype control Ab, or vehicle (Th2 and CEM cell analysis only), followed by flow cytometry analysis, as described in *Materials and Methods*. The data are representative of more than 3 different experiments with Th2 data representative of at least three different sets of differentiated cells.

Figure 3.3. The expression of PI3K isoforms and lipid phosphatases in cells exhibiting cell surface CCR4 expression.

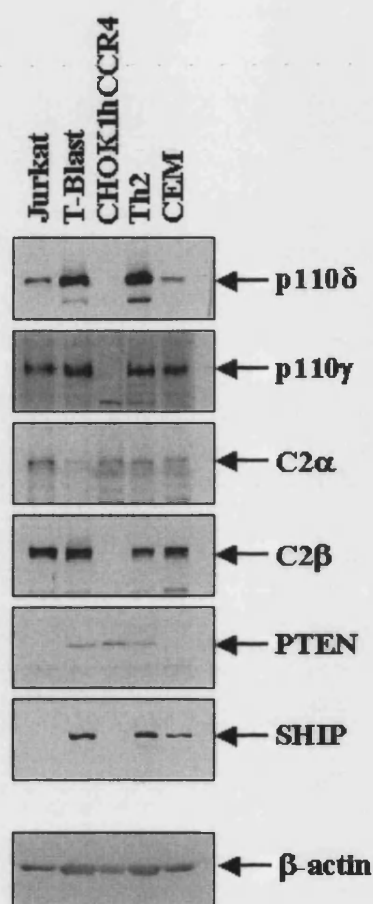


Figure 3.3. The expression of PI3K isoforms and lipid phosphatases in cells exhibiting cell surface CCR4 expression. Cells were washed and incubated in RPMI-1640 media, (or DMEM for CHOK1hCCR4 cells), containing 0.1% BSA and incubated at 37°C for 1 hr. Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted with the indicated antibodies, and protein was visualised by ECL, as described in *Materials and Methods*. The data are representative of at least three separate experiments. Blots were stripped and reprobed with β -actin to determine extent of equal loading as described in *Materials and Methods*, with the stripped and reprobed blot for p110 δ presented (which is also representative of the β -actin levels obtained for the other 5 blots).

Activation of the PI3K Pathway

Measurement of PKB Phosphorylation

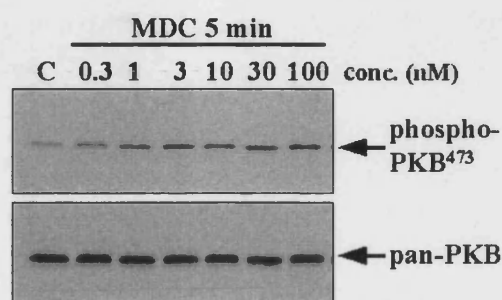
The major downstream effector of PI3K activity is PKB, a serine/threonine kinase, whose recruitment, phosphorylation and subsequent activation are entirely dependent upon the lipid products of PI3K (Vanhaesebroeck and Alessi, 2000), and consequently, is an indirect measure of PI3K activity. Several studies have reported the activation of PKB following stimulation of chemokine receptors other than CCR4 (Bonacchi et al., 2001; Smit et al., 2003; Sotsios et al., 1999), and PKB itself has been proposed to be involved in the polarisation of neutrophils responding to a chemotactic gradient (Hannigan et al., 2002; Servant et al., 2000). Thus, PKB phosphorylation can be monitored as an indirect measurement of PI3K activity.

CEM cells characteristically display a constitutive level of phosphorylated PKB due to the absence of the 3' – phosphoinositide lipid phosphatase PTEN (Freeburn et al., 2002). However, MDC stimulated phosphorylation of PKB (on Ser 473) above basal levels in a concentration-dependent manner (fig. 3.4, A). MDC-induced increases in PKB phosphorylation in a time-dependent manner with responses detectable at 30 sec post-stimulation reaching a maximum at 5 mins and returning toward basal levels at 30 min. (Fig. 3.4, B).

In order to determine if the PKB phosphorylation events witnessed in CEM cells are not a cell line specific phenomenon, various other CCR4-expressing cell lines, both T cell and non-T cell, were used. As was observed upon CCR4 ligation in CEM cells, MDC was capable of instigating the phosphorylation of PKB (on Ser 473) in all 3 CCR4-expressing cell types (fig. 3.5). Although this phosphorylation was time-dependent, the kinetics varied quite considerably between the different cell types. The leukaemic T cell line HUT-78 and the T-lymphoblasts exhibited similar kinetics, with the rapid (< 1 min) and transient (no detectable levels at 5 min) phosphorylation of PKB upon MDC stimulation (fig. 3.5, A and B). The CCR4 transfected CHOK1 cells however displayed a much slower onset of PKB phosphorylation (5 – > 30 min) (fig. 3.5, C). CCR4 untransfected CHOK1 parent cells do not signal in response to MDC or TARC (fig. 3.6).

Figure 3.4. MDC stimulates the phosphorylation of the PI3K effector PKB in a dose-dependent manner in CEM cells.

A



B

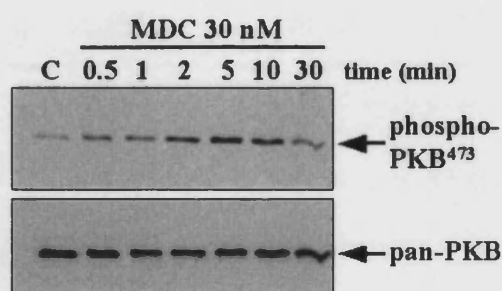


Figure 3.4. MDC stimulates the phosphorylation of the PI3K effector PKB in a dose-dependent manner in CEM cells. Levels of phospho-PKB protein were determined by western blot analysis. CEM cells were incubated in low serum (2% FBS) for 16 hrs at 2×10^6 cells/ml. Prior to experimentation, CEM cells were washed three times in RPMI, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with *A*, indicated concentrations of MDC for 5 mins, or *B*, MDC (30nM) for the indicated times, and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the Ser 473-phosphorylated, active form, of PKB, and protein was visualised with ECL. Stripping and reprobing blots with anti-PKB Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 3 separate experiments.

Figure 3.5. MDC stimulates the phosphorylation of PKB in various types of cells, with varying kinetics.

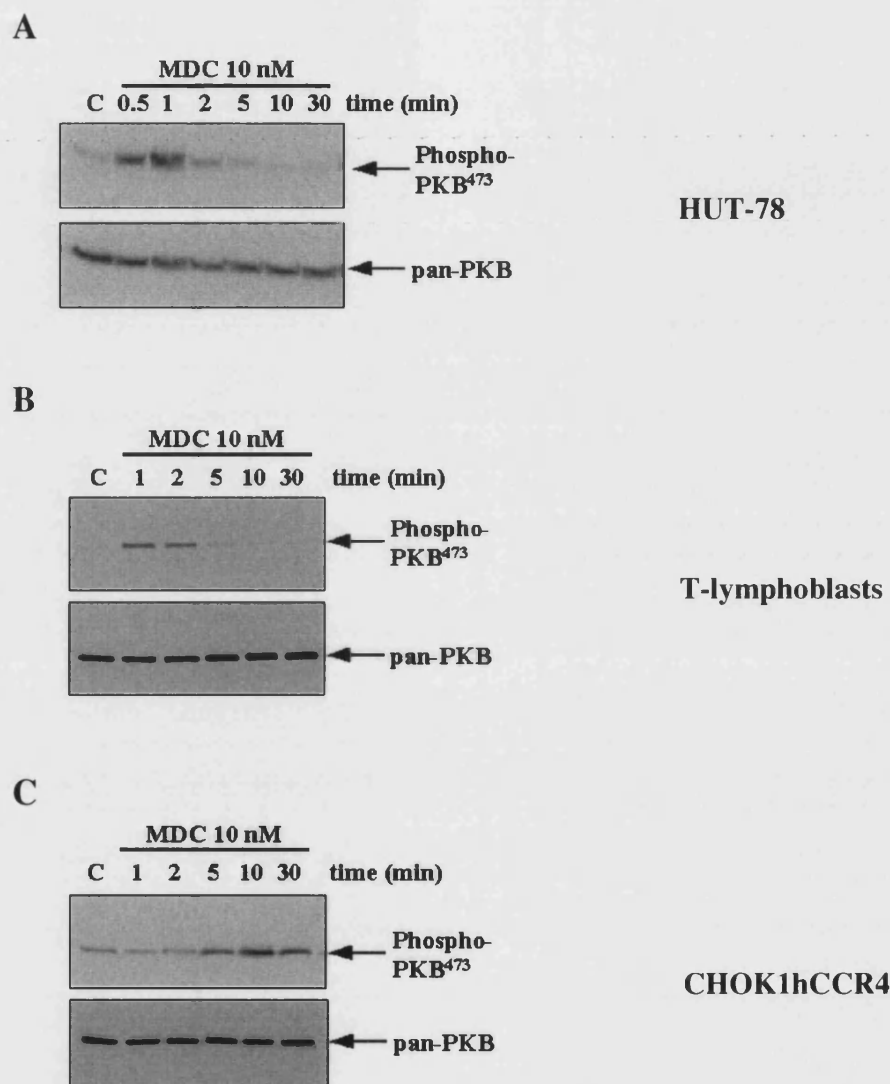


Figure 3.5. MDC stimulates the phosphorylation of PKB in various types of cells, with varying kinetics. Levels of phospho-PKB protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in media, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. Cells (A, HUT-78, or B, T-lymphoblast, or C, CHOK1hCCR4) were then stimulated at 37°C with MDC for indicated times, and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the Ser 473-phosphorylated, active form, of PKB, and protein was visualised with ECL. Stripping and reprobing blots with anti-PKB Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 3 separate experiments.

Figure 3.6. CHOK1 parent cells (i.e. CCR4 untransfected CHOK1 cells) do not signal in response to MDC or TARC.

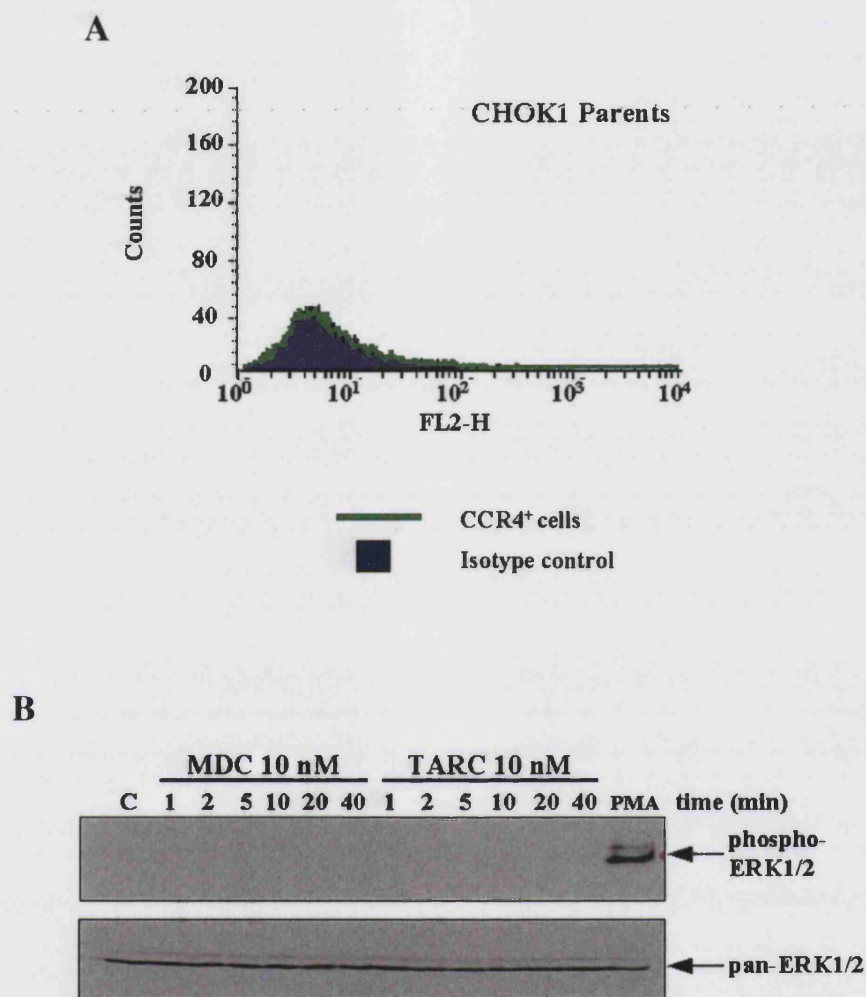


Figure 3.6. CHOK1 parent cells (i.e. CCR4 untransfected CHOK1 cells) do not signal in response to MDC. Expression of CCR4 was assessed by immunostaining 1×10^6 cells $1 \mu\text{g}$ of R-phycoerythrin-conjugated mouse anti-human CCR4 mAb (1G1), or PE-conjugated IgG $_{1,\kappa}$ isotype control Ab, followed by flow cytometry analysis, as described in *Materials and Methods*. Levels of phospho-ERK1/2 protein were determined by western blot analysis. CHOK1 were washed three times in DMEM, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC (10 nM) or TARC (10 nM) for indicated times, *B*, and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK1/2 Ab with affinity for the Thr 202/Tyr 204 phosphorylated, active form, of ERK, and protein was visualised with ECL. Stripping and reprobing blots with anti-ERK Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 2 separate experiments.

CCR4 Ligation Stimulates the Accumulation of PI(3,4,5)P₃ in CEM Cells

An alternative method of determining the extent of PI3K activation is to assess the accumulation of one of its lipid products, namely PI(3,4,5)P₃. Although the method of measuring this lipid is substantially more time-consuming and utilises large amounts of gamma radioactivity, it is a more direct measure of PI3K activity. Consequently, ³²P-labelled CEM cells were used to investigate the effect of MDC stimulation upon the levels of this phospholipid. Treatment of CEM cells with MDC led to a time-dependent increase in PI(3,4,5)P₃ levels, with a maximum response seen at 2 – 5 min (fig. 3.7).

Activation of PI3K Isoforms and their Sensitivity Towards PI3K Inhibitors

In order to further define the PI3K response it is necessary to establish the PI3K isoforms involved and the effect that the PI3K inhibitors employed in the remainder of this chapter have on the individual isoforms. To verify this, we ascertained whether immunoprecipitates of the various class I and II PI3K isoforms derived from MDC-stimulated CEM cells exhibited enhanced *in vitro* lipid kinase activity above that of unstimulated levels. GPCRs are known to be able to activate p110γ (PI3Kγ) directly via their associated Gβγ proteins. Accordingly, MDC stimulated an increase in the *in vitro* activity of p110γ that was immensely rapid and transient, with detectable stimulation occurring only at 30 sec (fig. 3.8, A). Additionally, immunoprecipitating the p85 regulatory/adaptor subunit of the class IA isoforms, (thereby assessing the activity of p110α, β and δ), demonstrated an increase in activity at around 1-2 mins (fig. 3.8, B), suggesting an increase in activity of one of the class IA isoforms. Furthermore, p110δ (an isoform shown to be required for neutrophil chemotaxis) was rapidly activated in response to MDC with an increase in activity observed at 1 min (fig. 3.8, C). Both the class IA and IB isoforms were sensitive to LY294002 and wortmannin with p110δ being particularly sensitive to wortmannin. The class II PI3K isoforms, C2α and C2β, showed no detectable levels of stimulation above those of the considerably high basal levels of activity (fig. 3.9, A and B). However, C2β basal kinase activity showed resistance to the PI3K inhibitor LY294002, with only a moderate level of inhibition at 30 μM, but concentration-dependent sensitivity to wortmannin. C2α demonstrated noticeable sensitivity to both LY294002 and wortmannin at the higher concentrations.

Figure 3.7. MDC stimulates the time-dependent accumulation of PI(3,4,5)P₃ in CEM cells.

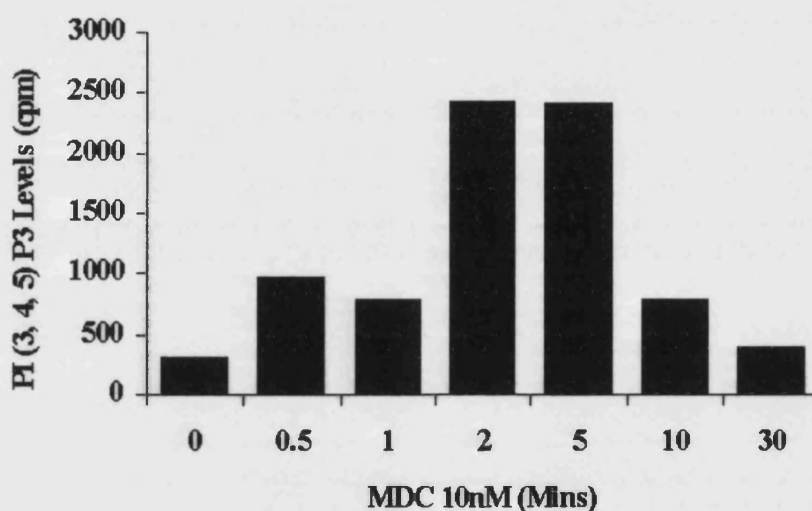
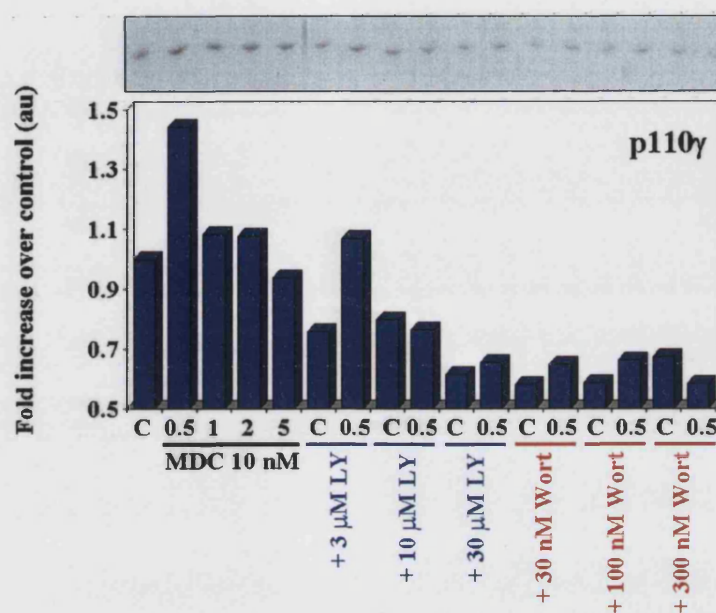


Figure 3.7. MDC stimulates the time-dependent accumulation of PI(3,4,5)P₃ in CEM cells. Lipid analysis was carried out using a total of 2×10^7 ³²P-labelled CEM cells that were stimulated at 37°C with MDC (10 nM) for indicated times. Phospholipids were extracted and deacylated with the glycerophosphorylinositol derivatives of PI(3,4,5)P₃ analysed by HPLC, as described under *Materials and Methods*. Data is representative of at least 2 separate experiments.

Figure 3.8. MDC-mediated CCR4 ligation in CEM cells leads to the activation of the Class I PI3K isoforms, p110 δ and p110 γ .

A



B

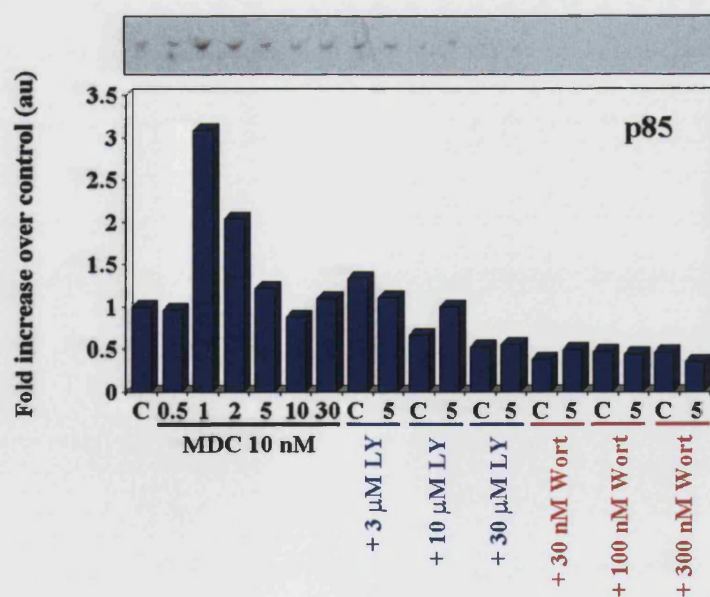


Figure 3.8. legend on following page.

Figure 3.8. continued.

C

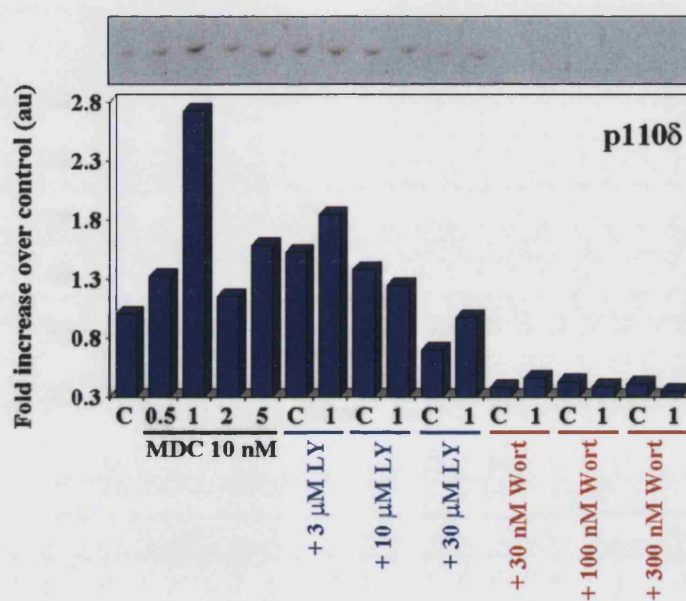
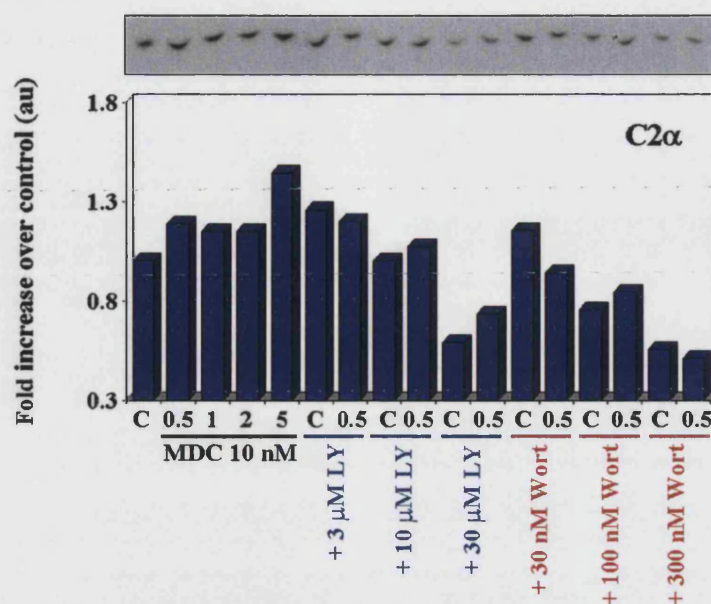


Figure 3.8. MDC-mediated CCR4 ligation in CEM cells leads to the activation of the Class I PI3K isoforms, p110δ and p110γ. A total of 1×10^7 CEM cells were stimulated at 37°C for various times (labels in min) with 10 nM MDC. Cells were lysed and lysates were subjected to immunoprecipitation with A, an anti-p110γ Ab, B, an anti-p85 Ab, and C, an anti-p110δ Ab. Immunoprecipitates were washed and, where indicated, treated with LY294002 or wortmannin (30 and 15 min, respectively), and analysed for PI kinase activity, as described in *Materials and Methods*. Lipids were detected by exposure to film at -70°C, and, additionally, analysed by densitometry to determine PI(3)P generation (graphs). Results are representative of at least two separate experiments. C = vehicle control (stimulated with vehicle for same length of time as MDC with LY/Wort treatment i.e. 1, 0.5 and 5 min for p110γ, p85 and p110δ, respectively).

Figure 3.9. Class II PI3K isoforms are not stimulated upon MDC stimulation of CEM cells.

A



B

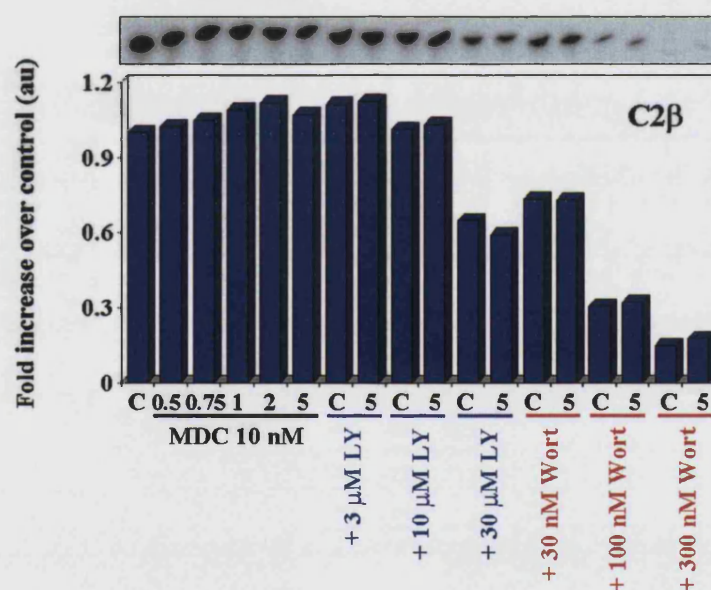


Figure 3.9. Class II PI3K isoforms are not stimulated upon MDC stimulation of CEM cells. A total of 1×10^7 CEM cells were stimulated at 37°C for various times with 10 nM MDC. Cells were lysed and lysates were subjected to immunoprecipitation with A, an anti-C2α Ab, and B, an anti-C2β Ab. Immunoprecipitates were washed and, where indicated, treated with LY294002 or wortmannin, and analysed for PI kinase activity, as described in *Materials and Methods*. Lipids were detected by exposure to film at -70°C, and, additionally, analysed by densitometry to determine PI(3)P generation (graphs). Results are representative of at least two separate experiments. C = vehicle control (stimulated with vehicle for same length of time as MDC with LY/Wort treatment i.e. 0.5 and 5 min for C2α and C2β, respectively).

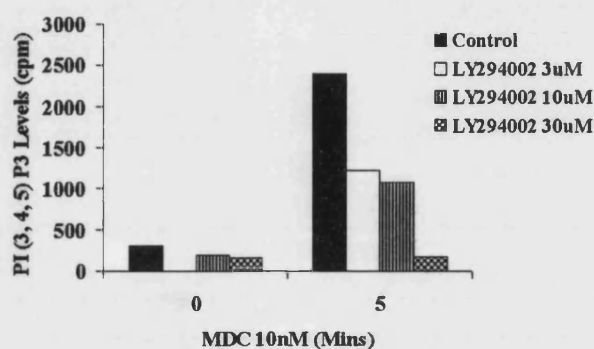
Regulation of PI3K Activity with Pharmacological Inhibitors

To confirm the inhibitory effects observed on individual PI3K isoform activity in IVLK assays (fig. 3.8 and 3.9) with the PI3K inhibitors are translated to *in vitro* lipid accumulation, the lipid-labelling assay was performed. Cells were labelled with ^{32}P -orthophosphate and pretreated with the chemically distinct PI3K inhibitors LY294002 (30 min) and wortmannin (15 min) at concentrations revealed to inhibit the kinase activity of all the tested PI3K isoforms. The importance of the inhibitors being chemically distinct is that they have different secondary target kinases (i.e. non-specific effects). This allows the elimination of possible non-PI3K activity inhibition on cellular events if both inhibitors demonstrate similar effects. MDC-stimulated $\text{PI}(3,4,5)\text{P}_3$ accumulation was diminished upon pretreatment with either LY294002 (fig. 3.10, A) or wortmannin (fig. 3.10, B) in a concentration-dependent manner, further confirming PI3K activity inhibition at the concentrations of PI3K inhibitors employed.

Additionally, phosphorylation of PKB (an additional marker of PI3K activation) on Ser 473 in response to MDC is abrogated with the use of LY294002 (fig. 3.11, A) in CEM cells. Furthermore, this effect was witnessed in the other CCR4-expressing cell types – HUT-78, T-lymphoblasts, and CHOK1 (fig. 3.11, B – D).

Figure 3.10. Accumulation of PI(3,4,5)P₃ is abrogated in a concentration-dependent manner with the use of PI3K inhibitors.

A



B

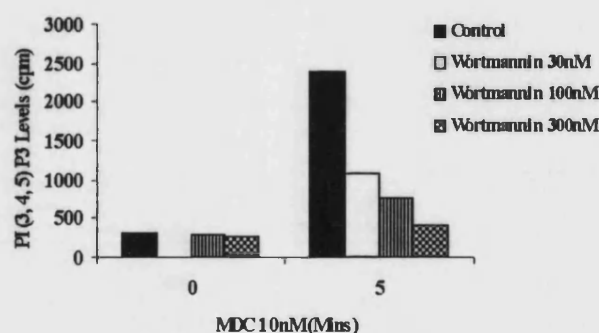


Figure 3.10. Accumulation of PI(3,4,5)P₃ is abrogated in a concentration-dependent manner with the use of PI3K inhibitors. A total of 2×10^7 ³²P-labelled CEM cells were stimulated at 37°C with A, pretreated for 30 mins with indicated concentrations of the PI3K inhibitor LY294002 before stimulation with MDC (10 nM) for 5 mins, and B, pretreated for 15 mins with indicated concentrations of the PI3K inhibitor wortmannin and subsequent stimulation with MDC (10 nM) for 5 mins. Phospholipids were extracted and deacylated with the glycerophosphorylinositol derivatives of PI(3,4,5)P₃ analysed by HPLC, as described under *Materials and Methods*. Data representative of at least 2 separate experiments.

Figure 3.11. Inhibition of MDC-stimulated PKB phosphorylation with LY294002 in a range of CCR4 expressing cells.

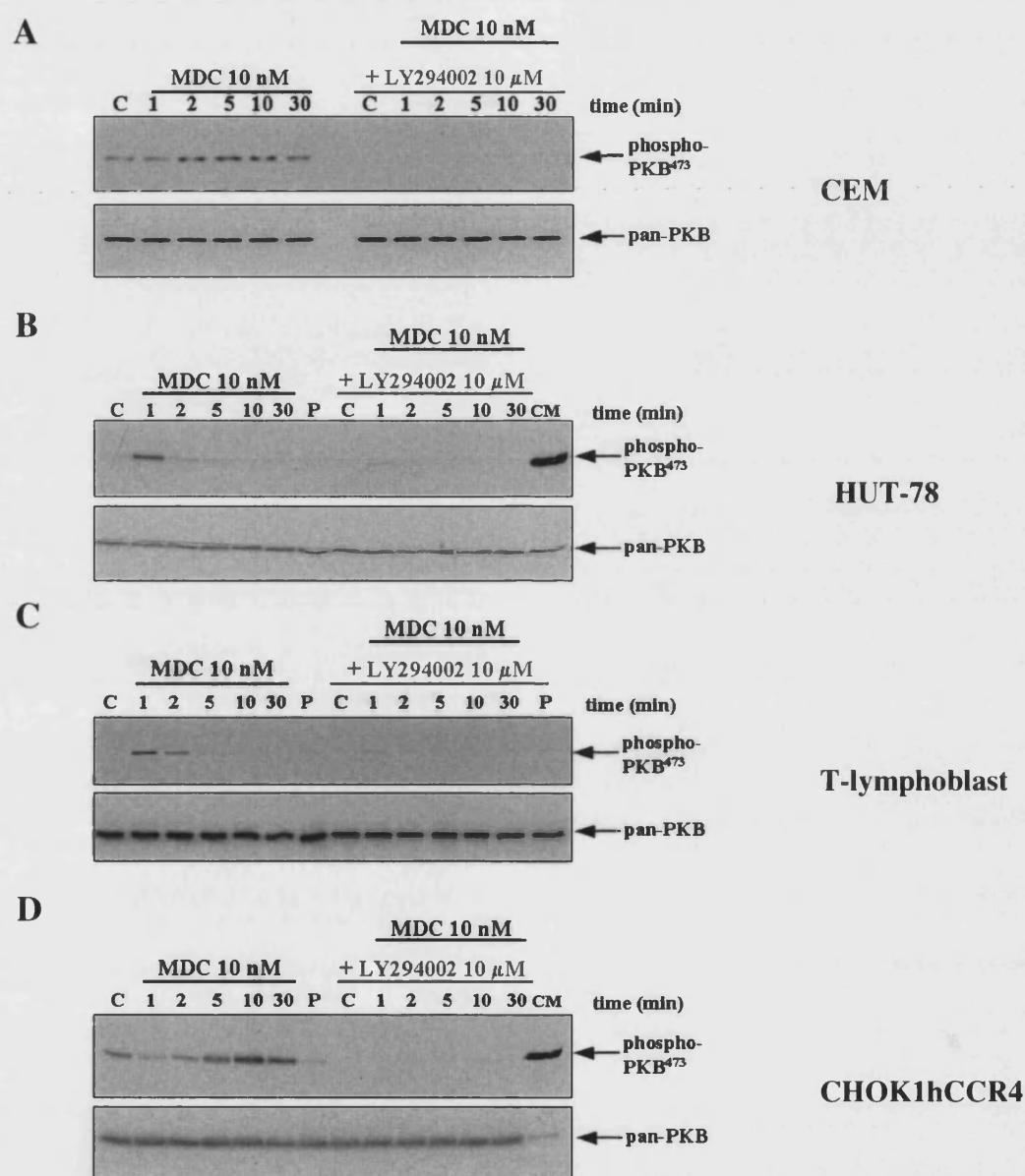


Figure 3.11. Inhibition of MDC-stimulated PKB phosphorylation with LY294002 in a range of CCR4 expressing cells. Levels of phospho-PKB protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in media, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. Cells (A, CEM, or B, HUT-78, or C, T-Lymphoblast, or D, CHOK1hCCR4) were then stimulated at 37°C with MDC for indicated times (in the presence or absence of LY294002, 30 min preincubation), and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the Ser 473-phosphorylated, active form, of PKB, and protein was visualised with ECL. Stripping and reprobing blots with anti-PKB Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 3 separate experiments. Key: P, 100 nM PMA 5 min; CM, CEM cell lysate sample.

Role of PI3K in Chemokine-Directed T Cell Migration

One functional response of a cell to activation with a chemokine is directed cell migration. Hence, we investigated whether MDC could stimulate chemotaxis of CEM cells. Several studies have demonstrated the ability of MDC to elicit the migration of a variety of CCR4 expressing cells (Chantry et al., 1999; Iellem et al., 2001; Imai et al., 1999). Accordingly, MDC stimulated the chemotaxis of CEM cells in a concentration-dependent manner with classic bell-shaped characteristics (fig. 3.12).

The involvement of PI3K in chemokine-mediated T cell chemotaxis has been reported for various receptors (Curnock et al., 2003; Hirsch et al., 2000; Sotsios et al., 1999). By means of pretreating the cells with LY294002 and wortmannin, we demonstrate that they have no negative impact upon MDC-mediated CEM cell chemotaxis, even at concentrations as high as 30 μ M and 300 nM, respectively (fig. 3.13, A). Yet there was a detrimental effect on basal migration with 30 μ M LY294002. However, when aliquots of the remaining (unused) treated cells were immunoblotted for the effect of MDC on pPKB Ser 473 there was an abrogation of the MDC stimulated phosphorylation of PKB with both LY294002 and wortmannin at the two highest concentrations (10 μ M, 30 μ M and 100nM, 300nM respectively) (fig. 3.13, B top blots). It has been suggested that phosphorylation at Thr 308 is required for full PKB activation (Scheid et al., 2002). Using an antibody that recognises PKB phosphorylation at Thr 308 there is a similar pattern to that observed with the pPKB Ser 473 Ab (fig. 3.13, B bottom blots). MDC had no effect on random migration (fig. 3.14).

Role of PI3K in Differentiated Human Th2 Cells

In order to determine whether the effects we are witnessing in a leukaemic T cell line are an accurate reflection of the events in peripheral blood-derived T lymphocytes, we examined CCR4 in Th2 cells, where expression of CCR4 is well documented (Bonecchi et al., 1998; D'Ambrosio et al., 1998; Zhang et al., 2000). As was observed in CEMs, MDC brought about increases in PKB phosphorylation in a time and concentration-dependent manner (fig. 3.15). However, the kinetics of PKB phosphorylation were more rapid and transient to that observed for MDC stimulation of CEM cells, with detectable increases in PKB Ser 473 phosphorylation occurring between 0.5-2 mins and returning to basal levels within 5 mins.

Figure 3.12. MDC stimulates the directed migration of CEM cells in a concentration-dependent manner.

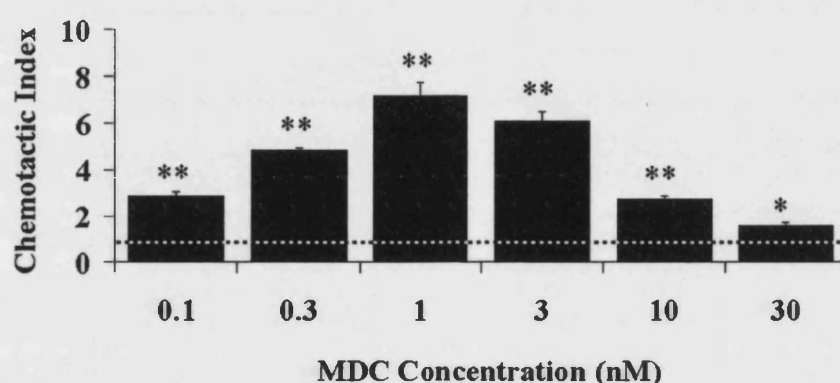
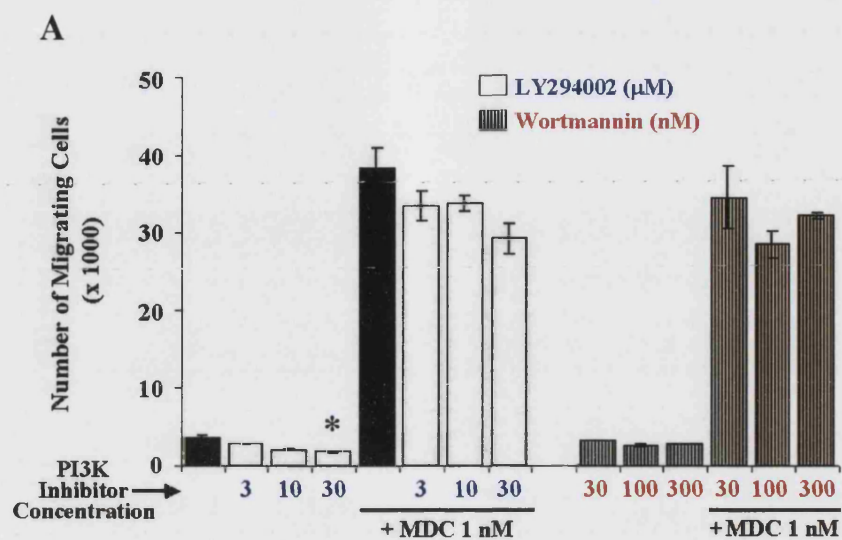


Figure 3.12. MDC stimulates the directed migration of CEM cells in a concentration-dependent manner. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing varying concentrations of MDC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test, with Bonferroni correction, for significance of MDC-stimulated migration compared with randomly migrating cells (*, $p < 0.0005$; **, $p < 0.0001$; ***, $p < 0.00005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Figure 3.13. MDC-stimulated CEM cell chemotaxis is insensitive to PI3K inhibitors.



Treatment	Vehicle	LY294002 (μM)			Wortmannin (nM)		
		3	10	30	30	100	300
Control	3650 ± 253	2820 ± 120	2125 ± 131	1825 ± 103	3233 ± 67	2500 ± 153	2750 ± 56
Significance (p value)	> 0.05	> 0.05	> 0.05	< 0.01	> 0.05	> 0.05	> 0.05
MDC 1 nM	38260 ± 2735	33475 ± 1892	33840 ± 1056	29333 ± 1968	34500 ± 4002	28500 ± 1745	32208 ± 423
Significance (p value)	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

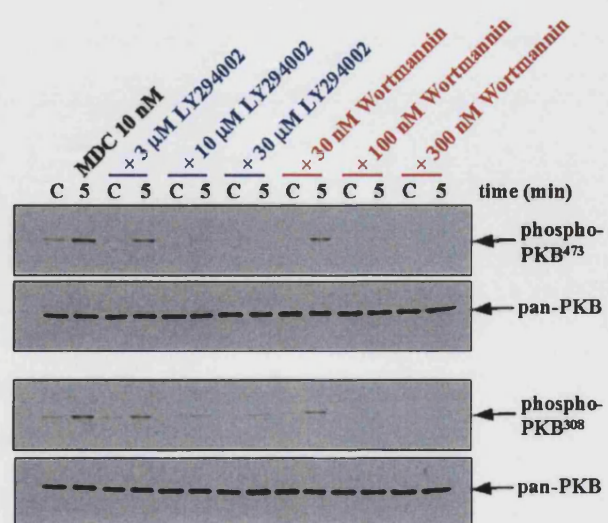
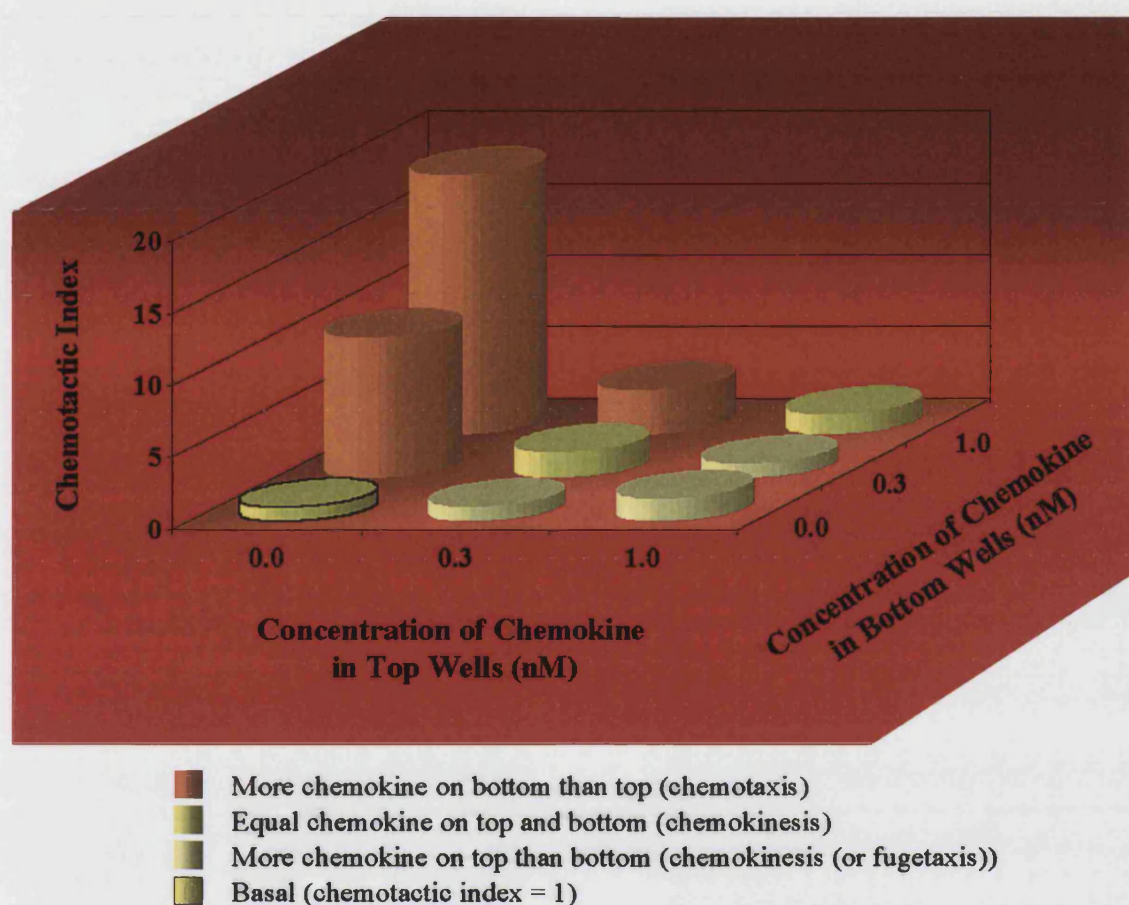
B

Figure 3.13. MDC-stimulated CEM cell chemotaxis is insensitive to PI3K inhibitors.

CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with the stated concentrations of LY294002 (LY) or wortmannin (wort) for 30 or 15 minutes respectively, *A*, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the number of cells migrated. Data were analysed by ANOVA and Student's *t* test, with Bonferroni correction, to compare responses in the presence and absence of PI3K inhibitors within control and MDC-stimulated groups (*, $p < 0.01$). A table of the actual values is presented below the graph for the reader to peruse. A sample of cells from those treated and untreated with the PI3K inhibitors for the chemotaxis assay was taken and stimulated with MDC (10 nM) for indicated times (C = vehicle 5 min), and lysates generated, *B*. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho PKB⁴⁷³ Ab and anti-phospho PKB³⁰⁸ Ab. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer.

Figure 3.14. MDC has no effect on chemokinesis in CEM cells.



Chemokine Concentration in Bottom Wells (nM)	Chemokine Concentration in Top Wells (nM)		
	0.0	0.3	1.0
0.0	1.00*	1.03	1.59
0.3	9.78	1.79	1.00
1.0	18.06	3.06	1.37

* = basal (chemotactic index)

Figure 3.14. MDC has no effect on chemokinesis in CEM cells. CEM cells (2×10^5 cells/200 μ l) incubated (5 min) with varying concentrations of MDC, as indicated, were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing varying concentrations of MDC alone, as indicated in the graphic. The assay was then performed according to the chemotaxis protocol detailed in *Materials and Methods*. Chemotaxis/chemokinesis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of two other experiments. Values in table represent the data displayed in the graph, presented as chemotactic index (taking 0 chemokine on top and bottom as basal i.e. chemotactic index of 1).

Figure 3.15. MDC stimulates the rapid and transient phosphorylation of the PI3K effector PKB in a concentration-dependent manner in human Th2 cells.

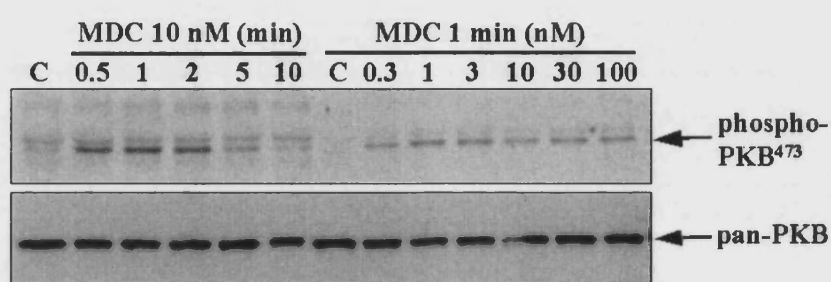


Figure 3.15. MDC stimulates the rapid and transient phosphorylation of the PI3K effector PKB in a concentration-dependent manner in human Th2 cells. Th2 cells were generated, as described in *Materials and Methods*, and prior to experimentation were washed three times in RPMI, and resuspended at 5×10^5 cells/500 μ l and serum-starved for 1 hour. Cells were then stimulated at 37°C with indicated concentrations of MDC for 1 min, or MDC (10nM) for the indicated times and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the Ser⁴⁷³-phosphorylated form of PKB, and protein was visualised with ECL. Stripping and immunoblotting with an anti-PKB Ab, as described in *Materials and Methods*, confirmed equal loading.

Th2 cells exhibit the same migratory characteristics as those of CEM cells towards MDC

MDC stimulated the chemotaxis of Th2 cells in a dose-dependent fashion with a maximum migration reached at 50-100 nM (fig. 3.16, A). LY294002 and wortmannin treated Th2 cells were still wholly responsive to MDC when monitoring chemotaxis (fig. 3.17, A), yet the phosphorylation of PKB^{Ser473} was prohibited at similar concentrations by the PI3K inhibitors LY294002 and wortmannin (fig. 3.16, B, and 3.17, B).

Do Both CCR4 Ligands Act in a Comparable Manner with Respect to PI3K?

The CCR4 receptor has an additional ligand to that of MDC, namely TARC. Akin to MDC, TARC exclusively binds CCR4 suggesting that there must be differences between the two chemokines in their functional roles, and therefore signalling through CCR4. However, with regard to PI3K, TARC appears to display similar properties to MDC. TARC is able to stimulate the phosphorylation of PKB with similar kinetics as MDC (fig. 3.18). However, the extent of phosphorylation is not as high as observed with MDC. Furthermore, TARC-mediated chemotaxis of Th2 cells is insensitive to pretreatment with LY294002 (identical to MDC) and displays a similar ability to MDC in the extent of cell migration achieved (fig. 3.19, B, and fig. 3.20). Conversely, TARC is unable to stimulate the directed migration of CEM cells to anywhere near the same extent as to that observed with MDC (fig. 3.19, A), and consequently, there is difficulty in examining the effects of inhibitors upon CEM cell migration in response to TARC (max. chemotactic index of 2).

A PKB Inhibitor has no Effect on CEM Cell Chemotaxis

The PKB inhibitor PKBi (1L-6-Hydroxymethyl-*chiro*-inositol 2-[(R)-2-O-methyl-3-O-octadecylcarbonate]) has been demonstrated to have 40-fold selectivity for PKB (IC₅₀ = 2 µM) over PI3K (IC₅₀ = 83 µM) (Hu et al., 2000). As would be expected from the lack of effect with PI3K inhibition, 10 µM PKBi pretreatment had no detrimental impact on CEM chemotaxis mediated by MDC (fig. 3.21), although PKB kinase assays are required to confirm the compound is having the desired inhibitory effect on PKB.

Figure 3.16. MDC induces the concentration-dependent directed migration of Th2 cells.

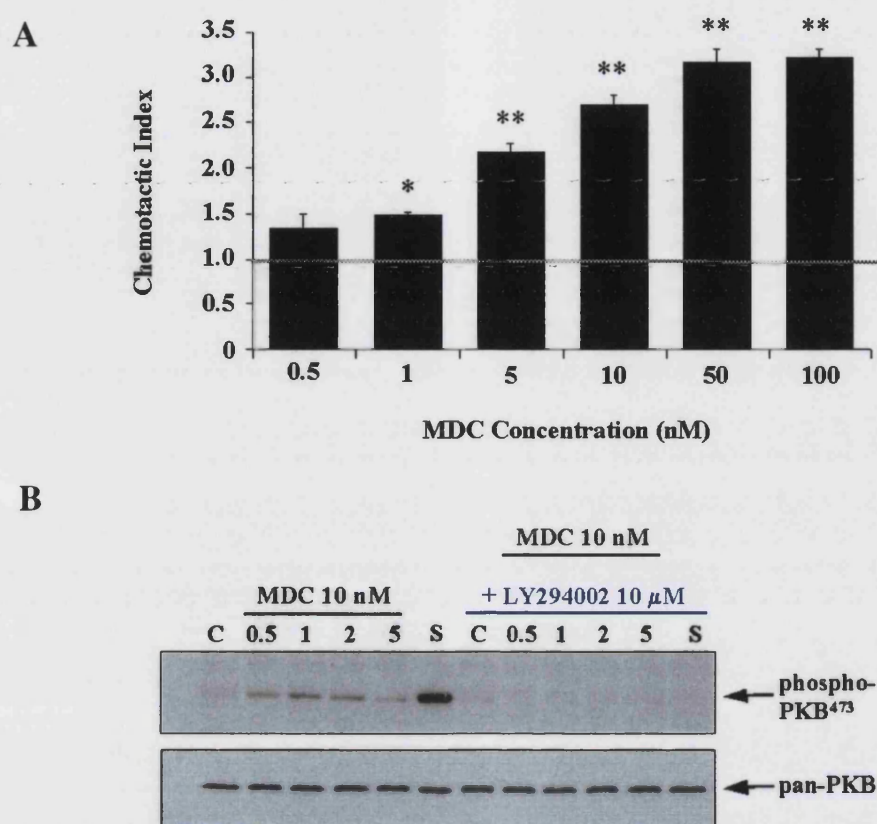


Figure 3.16. MDC induces the concentration-dependent directed migration of Th2 cells. Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing varying concentrations of MDC, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test, with Bonferroni correction, to compare MDC-induced migration to basal (*, $p < 0.05$; **, $p < 0.001$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Western blot analysis was performed by resuspending Th2 cells at 5×10^5 cells/500 μ l and then serum-starved for 1 hour. Cells were then stimulated at 37°C with 10 nM MDC for indicated times in the presence or absence of LY294002 (30 min preincubation) and lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the Ser⁴⁷³-phosphorylated form of PKB, and protein was visualised with ECL. Stripping and immunoblotting with an anti-PKB Ab, as described in *Materials and Methods*, confirmed equal loading. S = SDF-1/CXCL12 10 nM, 1 min (positive control).

Figure 3.17. LY294002 and wortmannin display no consequence on MDC-induced Th2 cell chemotaxis, but attenuate the MDC-mediated phosphorylation of PKB at Ser⁴⁷³.

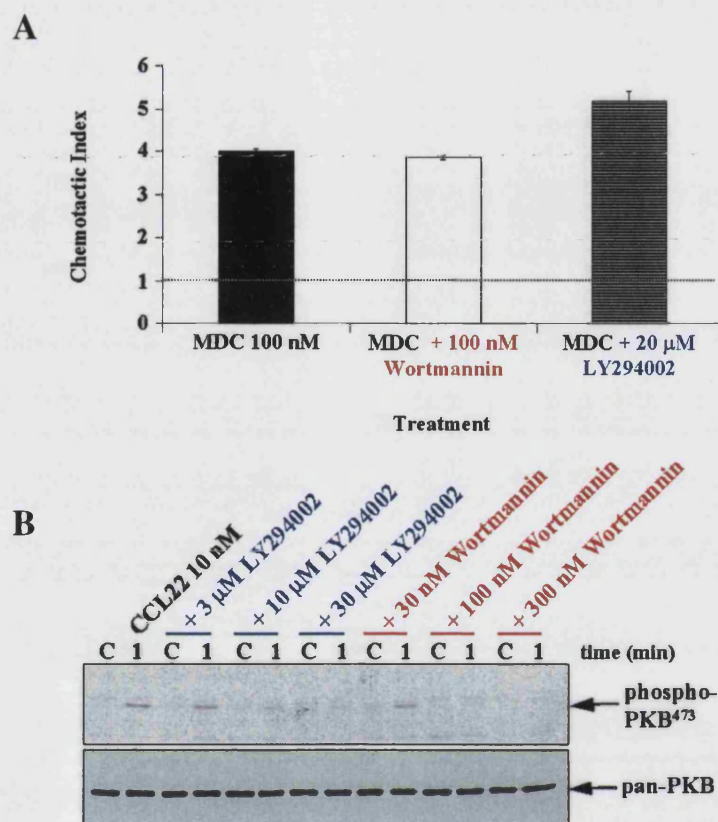


Figure 3.17. LY294002 and wortmannin display no consequence on MDC-induced Th2 cell chemotaxis, but attenuate the MDC-mediated phosphorylation of PKB at Ser⁴⁷³. Th2 cells were washed and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Where indicated, *graph A*, cells were preincubated with varying concentrations of LY294002 (LY) or wortmannin (wort) for 30 or 15 minutes respectively. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing MDC (100 nM), as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. *B*, Th2 cells were washed three times in RPMI, and resuspended at 5×10^5 cells/500 μ l and serum-starved for 1 hour. As indicated, cell samples were pretreated with either LY294002 or wortmannin for 30 or 15 mins, respectively. Cells were then stimulated at 37°C with MDC (10nM) for 1 min or with vehicle for 1 min (C), before cells were lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phosphoPKB^{Ser473}, and protein was visualised with ECL. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer.

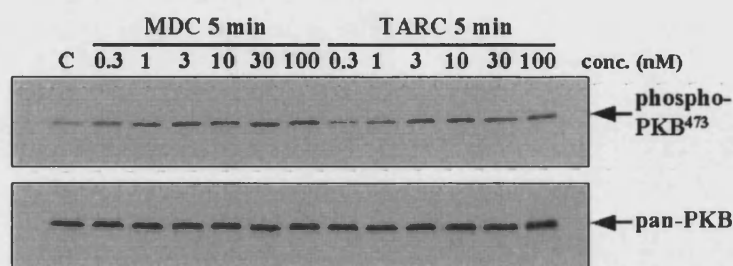
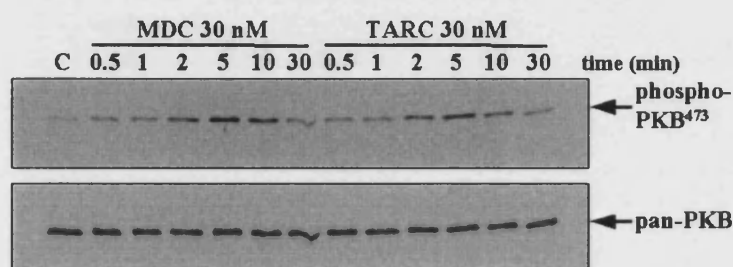
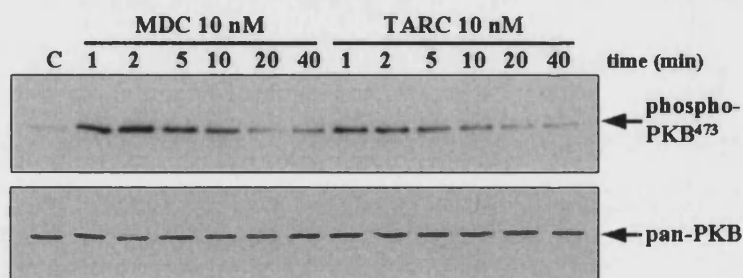
Figure 3.18. TARC stimulates the phosphorylation of PKB.**A****CEM****B****T-lymphoblast**

Figure 3.18. TARC stimulates the phosphorylation of PKB. Levels of phospho-PKB protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in media, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. Cells (A, CEM, or B, T-Lymphoblast) were then stimulated at 37°C with TARC or MDC (10 or 30 nM) for indicated times, or for 5 min with indicated concentrations, and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific PKB Ab with affinity for the Ser 473-phosphorylated, active form, of PKB, and protein was visualised with ECL. Stripping and reprobing blots with anti-PKB Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 2 separate experiments.

Figure 3.19. TARC is able to chemotax Th2 cells in a concentration-dependent manner.

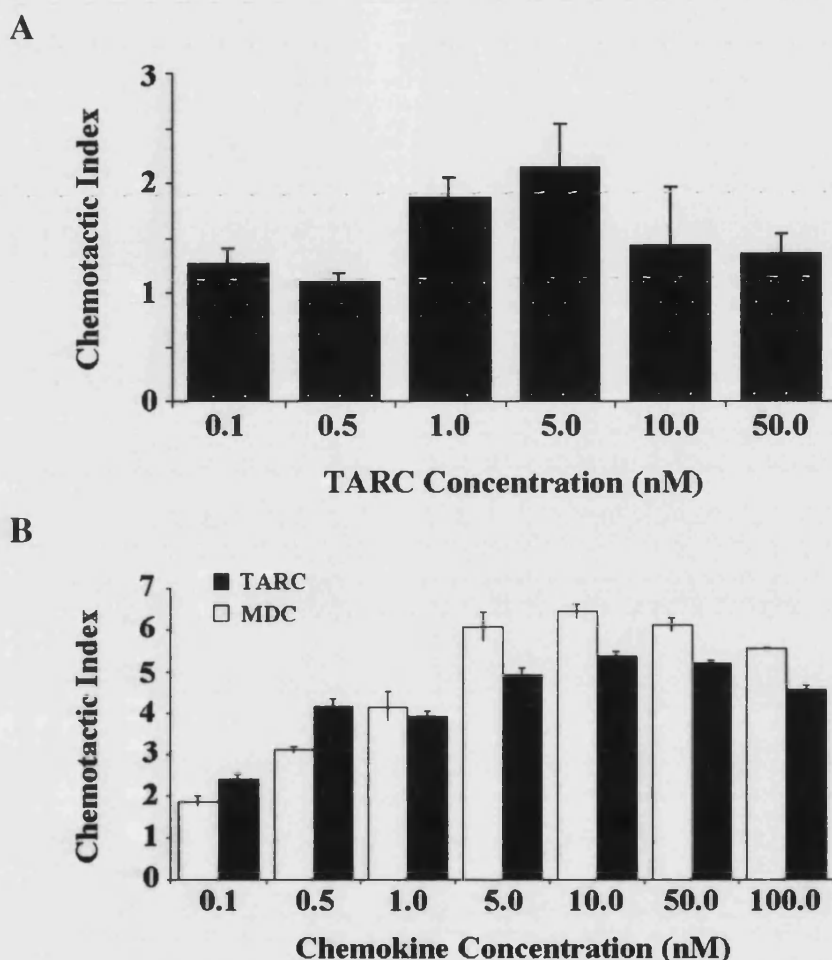


Figure 3.19. TARC is able to chemotax Th2 cells in a concentration-dependent manner. *A*, CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing varying concentrations of TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward TARC vs. cells randomly migrating. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. *B*, Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing varying concentrations of MDC or TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC/TARC vs. cells randomly migrating.

Figure 3.20. TARC-mediated Th2 cell chemotaxis is unaffected by PI3K inhibition.

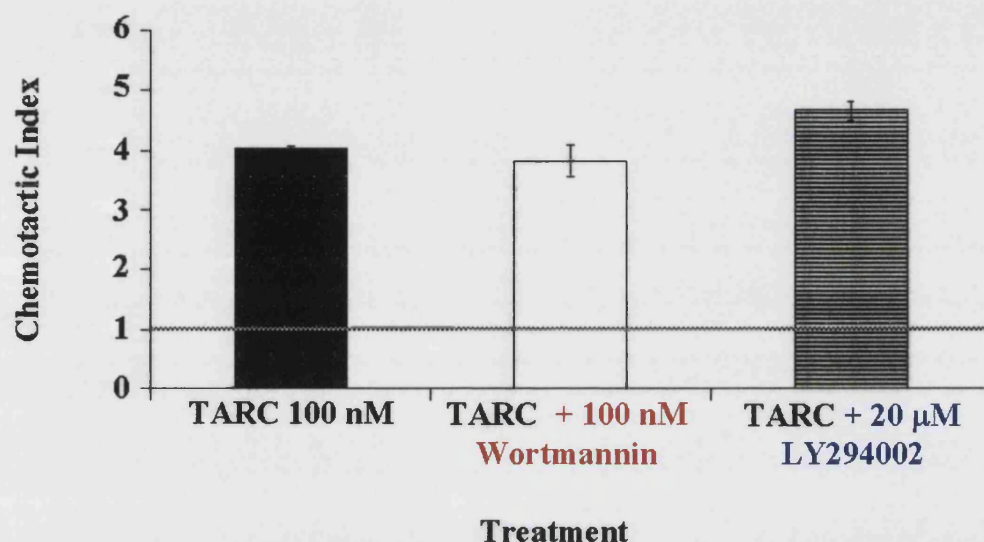


Figure 3.20. TARC-mediated Th2 cell chemotaxis is unaffected by PI3K inhibition.

Th2 cells were washed and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Where indicated, *graph A*, cells were preincubated with varying concentrations of LY294002 or wortmannin for 30 or 15 minutes respectively. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing TARC (100 nM), as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward TARC vs. cells randomly migrating (+/- inhibitor). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Data were analysed by Student's *t* test to compare responses in the presence and absence of PI3K inhibitor with Bonferroni correction ($p < 0.05$).

Figure 3.21. Sensitivity of CCR4-mediated CEM cell chemotaxis to a PKB inhibitor.

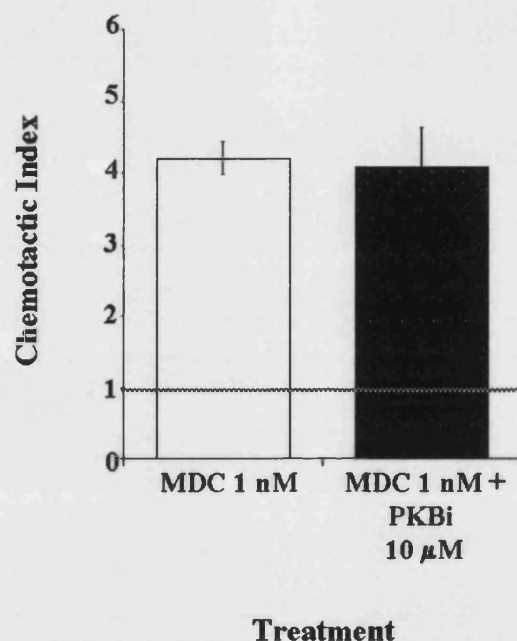


Figure 3.21. Sensitivity of CCR4-mediated CEM cell chemotaxis to a PKB inhibitor.

CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with 10 μ M PKBi for 3 hours, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test to compare responses in the presence and absence of inhibitor ($p < 0.05$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Summary

- CCR4 is expressed to varying degrees in a number of transformed and non-transformed T cells. In addition, untransfected CHOK1 cells fail to signal in response to either MDC or TARC, unlike the CCR4 transfected CHOK1 cells.
- All of the tested T cell types express the PI3K isoforms p110 δ , PI3K γ , C2 α and C2 β . However, CHOK1hCCR4 cells lack p110 δ , PI3K γ and C2 β . The HUT-78 T cell line along with T-lymphoblasts and Th2 cells express both PTEN and SHIP lipid phosphatases. The leukaemic T cell line CEM lacks PTEN and CHOK1hCCR4 cells lack SHIP. Jurkats lack both SHIP and PTEN.
- Ligation of CCR4 with MDC results in the phosphorylation of PKB in a concentration-dependent manner. The kinetics of this chemokine-mediated event varied between the different cell types, from rapid and transient to a slower onset and more prolonged PKB phosphorylation. This phosphorylation was sensitive to PI3K inhibition.
- MDC stimulation of CEM cells leads to the PI3K-dependent accumulation of PI(3,4,5)P₃. Furthermore, CCR4 activation results in the increased activity of all the class I (both I_A and I_B) PI3K isoforms tested for, but there were no detectable increases in the activity of class II PI3K isoforms. However, the kinase activities of all these PI3K isoforms were sensitive, to varying degrees, to both LY294002 and wortmannin.
- The concentration-dependent MDC-mediated directed CEM cell migration was insensitive to both LY294002 and wortmannin.
- Th2 cells behave in a similar manner to CEM cells with regard to CCR4-mediated events: MDC is able to activate the PI3K-PKB pathway and elicit the PI3K-independent chemotaxis of Th2 cells.
- Studies performed to date indicate that TARC behaves in a similar manner to MDC pertaining to the role of PI3K in directed T cell migration.

3.2 Discussion I

PI3K Involvement in CCR4 Signal Transduction and Chemotaxis

This section has investigated the role of PI3K in the regulation of MDC/CCR4-mediated PKB phosphorylation and chemotaxis of T cells. In addition, a brief investigation into the comparison of TARC with MDC was also performed. With the use, principally, of human Th2 cells and the leukaemic T cell line CEM, it has been demonstrated that MDC-mediated CCR4 ligation leads to the activation of class IA and IB PI3K isoforms with the subsequent accumulation of $\text{PI}(3,4,5)\text{P}_3$ and phosphorylation of the major PI3K effector PKB. However, with the use of two chemically distinct PI3K inhibitors, wortmannin and LY294002, used at concentrations that were observed to inhibit the kinase activity of all the tested PI3K isoforms, as well as $\text{PI}(3,4,5)\text{P}_3$ accumulation and PKB phosphorylation (at both Thr 308 and Ser 473 residues), there is no negative effect on MDC-mediated Th2 or CEM cell chemotaxis. Furthermore, studies with TARC have indicated that this phenomenon of PI3K-independent CCR4-mediated T cell chemotaxis is not restricted to just that migration induced by MDC. Although it appears that neither CEM cells nor Th2 cells require PI3K activity for chemotaxis it would seem that there are some fundamental differences between their responses to the CCR4 ligands.

Disparate PKB Phosphorylation Profiles of the Various Cell Types

The kinetics for PKB phosphorylation are far more rapid and transient in Th2 cells, as well as in HUT-78 and T-lymphoblasts, than those observed in CEM and CHOK1hCCR4 cells. The most logical explanation for this observation is the cellular presence or absence of the lipid phosphatases PTEN and SHIP. The inositol lipid 3-phosphatase PTEN, which hydrolyses $\text{PI}(3,4,5)\text{P}_3$ to $\text{PI}(4,5)\text{P}_2$, has been demonstrated to be absent from a variety of leukaemic T cell lines, including CEM (Astoul et al., 2001; Freeburn et al., 2002; Shan et al., 2000), and this has been confirmed here. In addition, it has been established that the lipid 5-phosphatase SHIP, which hydrolyses $\text{PI}(3,4,5)\text{P}_3$ to $\text{PI}(3,4)\text{P}_2$, is absent from the Jurkat T leukaemic cell line, but present in CEM cells (Freeburn et al., 2002). Further to this, it has been determined within the results presented here that CHOK1 cells lack SHIP,

not entirely unexpected due to the fact it is considered to be exclusively expressed by haemopoietic cells (Damen et al., 1996; Liu and Dumont, 1997), unlike SHIP2 (Pesesse et al., 1997; Sly et al., 2003). The cell types that express both PTEN and SHIP (T-lymphoblasts, HUT-78 and Th2 cells) display rapid and transient phosphorylation of PKB, whereas the cells lacking PTEN (CEM) or SHIP (CHOK1) demonstrate a much slower onset of PKB phosphorylation as well as this being more persistent. Moreover, the cells lacking PTEN display high basal levels of phosphorylated PKB, consistent with that seen in Jurkat cells (Curnock et al., 2003). Cells possessing PTEN had very low or undetectable levels of basal PKB phosphorylation. PTEN is required to 'turn off' the PI3K response by attenuating the levels of D3-phosphorylated inositol lipids. Thus, in the PTEN-expressing cell types, MDC (and TARC) is able to elicit the phosphorylation of PKB that is readily detected due to the low basal levels and this response is 'turned off' by the activity of PTEN. However, in PTEN deficient cells (e.g. CEM), there is likely a build-up of D3-phosphorylated lipids that eventually leads to the detectable increase in PKB phosphorylation that is more persistent due to the inability to down-regulate the 3-phosphoinositide lipids. Yet, the levels of phospho-PKB are reduced relatively quickly compared to those levels stimulated by SDF-1/CXCL12 (CXCR4) that are very prolonged (Curnock et al., 2003). This may be due to the presence of SHIP in CEM cells in which its activation leads to the degradation of $PI(3,4,5)P_2$ and therefore the accumulation of $PI(3,4)P_2$ (due to absence of PTEN), and subsequently curtail PKB activity.

Though there is some controversy over the lipid products required for PKB phosphorylation, centering on whether $PI(3,4)P_2$ or $PI(3,4,5)P_3$ is the critical substrate required for cellular localisation. There are studies suggesting that $PI(3,4)P_2$ is the principal substrate for the PH domain of PKB (Franke et al., 1997; Frech et al., 1997; Klippel et al., 1997), and this would correlate well with the observation in CEM cells of high basal phospho-PKB levels. The likelihood is that the presence of both SHIP and PTEN are required for the sufficient regulation of PKB activity, and studies have demonstrated that SHIP appears to play at least some role in the regulation of PI3K effectors (Freeburn et al., 2002; Isakoff et al., 1998; Lemmon and Ferguson, 2000). Although CHOK1 cells lack SHIP but possess PTEN and display significant basal phospho-PKB levels seemingly confirming a role of SHIP in PKB regulation, this is perhaps not the case and is due to experimental conditions. CHOK1 cells are adherent cells

but were stimulated in this study as a suspension cell therefore possibly increasing the levels of phospho-PKB due to stress and other such factors. Moreover, this cell type does not normally express SHIP (unlike the cell lines mentioned above with regard to PTEN) and thus it is probable that PKB activity is regulated via other phosphatases (either lipid e.g. SHIP2, or PKB-specific phosphatases (incidentally, this may also be the case in the T cell lines mentioned above)). The prolonged phosphorylation in CHOK1 cells of PKB in response to CCR4 ligation is likely to be due to many factors, with perhaps the biggest factor being the introduction of a receptor not normally expressed in this cell type. The introduction of a receptor to a cell by stable transfection does not ensure that the receptor couples to downstream signalling pathways in a correct manner. This is why there is always the requirement to carry out studies in cell types endogenously expressing the receptor of choice. A transfected cell line is, however, useful in indicating the possible signalling pathways that are activated in endogenously CCR4 expressing cells, with the added benefit of determining the authenticity of the response. This was highlighted by the inability of CHOK1 parent cells (untransfected with CCR4) to signal in response to MDC or TARC. This confirms the responses observed in CHOK1hCCR4 cells upon MDC/TARC stimulation are solely generated by ligated CCR4.

In addition to the differences between the cell types utilised in this study with regard to the expression of lipid phosphatases, there are also the variances observed in PI3K isoform expression to take into consideration. CHOK1 cells lacked p110 δ , p110 γ and C2 β PI3K isoforms that the T cells all possessed. This is not unexpected, as p110 δ and PI3K γ (p110 γ) are known to be leukocyte restricted (although recent studies suggest they may be more broadly expressed than first thought), however, C2 β is widely distributed and its absence slightly more surprising (Chantry et al., 1997; Sawyer et al., 2003; Vanhaesebroeck et al., 1997b). The remaining class I α isoforms (α and β) are widely expressed and essential for survival (El Sheikh et al., 2003; Sasaki et al., 2002). The upshot of these observations is that the delayed PKB phosphorylation in CHOK1hCCR4 cells may be due to the restricted PI3K isoform availability for the chemokine receptor to activate. PI3K γ is the sole PI3K isoform activated by GPCR and, as demonstrated here (although weakly), to be activated by chemokine receptors (Brock et al., 2003; Jones et al., 2003; Leopoldt et al., 1998). Likewise, p110 δ has been demonstrated, both here and in other systems, to be activated

downstream of chemokine receptors, although its activation is not restricted to GPCRs (Jou et al., 2002; Okkenhaug et al., 2002; Sadhu et al., 2003b). The lack of these two isoforms in CHOK1 cells is likely to be significant. While highly speculative, the rapid phosphorylation of PKB witnessed in T-lymphoblasts and Th2 cells may in part be due to their apparent hefty levels of p110 δ expression compared to the CEM and Jurkat cell lines. The IVLK assays are hard to derive any conclusive evidence about the extent and kinetics of PI3K isoform activation due to the high basal levels of activity but p110 δ does appear to be substantially activated upon MDC stimulation in CEM cells. The limitations of the IVLK data are two-fold: firstly, the substrate used is PI. Thus the generated product is PI(3)P and therefore not necessarily an accurate indicator regarding the generation of the lipids PI(3,4)P₂ and PI(3,4,5)P₃; secondly, the lipid generation is not measured within the cell, removing factors such as access to target lipid substrates. Despite these constraints, the IVLK assay is useful in determining a general increase in individual PI3K isoform activity and sensitivity to PI3K inhibitors (at least those that compete at the ATP-binding site). This clarification may help to explain the rapid and transient kinetics witnessed with CEM cells in IVLK assays yet the more latent kinetics observed with both PI(3,4,5)P₃ accumulation in lipid labelling studies and the phosphorylation of PKB. An additional observation is the high basal activity displayed by each of the PI3K isoforms in CEM cells (and also observed in Jurkat cells – general data from Ward group). This high kinase activity compared to ‘normal’ cells, such as T-lymphoblasts, cannot necessarily be accounted for by defects in the cells ability to regulate D3 PI lipids. It is possible that there may be a positive feedback mechanism occurring where increases in D3 PIs leads to recruitment of PH domain-containing proteins that are then able to further activate PI3K. This process will continue, and eventually reaching equilibrium, unless the D3 PIs are negatively regulated, which cannot occur if crucial lipid phosphatases are missing, resulting in abnormally high basal PI3K activity.

An alternative mechanism of PI3K activity regulation by PTEN is through direct management of upstream activators. It has recently been demonstrated that PTEN regulates PDGF receptor (and possibly other receptors) signalling through direct interaction, and dephosphorylates the activated tyrosine phosphorylated receptor and subsequent activation of downstream substrates (Mahimainathan and Ghosh, 2004). PTEN has displayed the

ability to dephosphorylate a number of proteins, including Shc and FAK in integrin-activated T cells (Gu et al., 1999; Leslie et al., 2000; Myers et al., 1997; Tamura et al., 1998; Tamura et al., 1999). Furthermore, there was evidence to suggest that PTEN may negatively regulate PDGF receptor in the absence of ligand (through constitutive association via PTEN's C2 domain), and upon ligand binding to the receptor PTEN dissociates allowing tyrosine phosphorylation and activation of receptor and substrates to occur (Mahimainathan and Ghosh, 2004). Therefore, in the absence of PTEN these PI3K activation pathways remain, at least in part, unregulated and thus cells lacking PTEN protein exhibit increased basal PI3K activity. PTEN may be regulated in normal circumstances through a feedback mechanism. One proposed model suggests that the elevated PI(3,4,5)P₂ levels activate a kinase (maybe PKB or CKII (casein kinase II)) that phosphorylates PTEN at Ser 380 and Ser 385 (and possibly threonine residues as well) in the C-terminal 'tail' and protects the protein from degradation (Birle et al., 2002; Torres et al., 2003; Torres and Pulido, 2001; Vazquez et al., 2000). When the PI(3,4,5)P₃ levels are reduced to basal amounts there is reduced PTEN phosphorylation and increase in PTEN degradation. A small amount of basal PI3K activity is required to maintain cell viability.

Activation of PI3K is Redundant for CCR4-Mediated T Cell Migration

In the past few years there has been an escalating body of evidence implicating a role, to varying degrees, for the PI3K family of enzymes in the directional migration of leukocytes/*Dictyostelium* in response to a chemokine gradient (Vanhaesebroeck et al., 1999). The class IA isoforms have been shown to have a role in SDF-1-mediated Jurkat chemotaxis (Curnock et al., 2003) and essential for *Dictyostelium* migration (Funamoto et al., 2002; Iijima and Devreotes, 2002). Recently, the development of a selective p110 δ isoform inhibitor has led to the seemingly essential role of this isoform in neutrophil chemotaxis (Sadhu et al., 2003b; Sadhu et al., 2003a) and involvement in breast cell migration (Sawyer et al., 2003). However, the use of p110 γ ^{-/-} mice has also implicated a role of this class IB isoform in neutrophil (and macrophage) chemotaxis (Hannigan et al., 2002; Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000), although migration is not completely attenuated (50-90%), and with a surprising lack of effect on lymphocyte migration. This suggests either another isoform (p110 δ ?) or a PI3K-independent pathway

may additionally be involved (Chodniewicz and Zhelev, 2003a; Chodniewicz and Zhelev, 2003b). Despite the development of PI3K isoform knockout mice and the generation of PI3K inhibitors (some isoform specific), studies have primarily concentrated on the chemotaxis of granular leukocytes and *Dictyostelium*. In spite of this, there is increasing evidence to suggest that perhaps PI3K isoforms do not provide the directional cues for T lymphocyte migration. A recent study by Smit, et al. (Smit et al., 2003) demonstrated that CXCR3 chemotaxis of PBMCs in response to CXCL11/I-TAC was insensitive to either LY294002 or wortmannin at concentrations up to 30 μ M and 300 nM, respectively. These chemotaxis results display a similar resistance to the PI3K inhibitors as the data presented within this thesis. Additionally, PI3K inhibitors had little effect on *in vivo* T lymphocyte arrest and adhesion to high endothelial venules in exteriorised Peyer's patches (Constantin et al., 2000) or on the SDF-1(CXCL12) and ELC (CCL19)/SLC (CCL21) transendothelial migration in laminar flow chambers (Cinamon et al., 2001). Smit has suggested that perhaps class II PI3K isoforms may still be involved. Although we demonstrate activation of class IA and IB isoforms in T cells with MDC, we observe no stimulation of the class II isoforms. This may well be due to the inability of the assay to reliably detect any stimulated increases above the considerably high basal levels witnessed in these cells. There is currently no published data that has ascertained the presence of C2 β downstream of activated chemokine receptors. Contrastingly, C2 α has been demonstrated to be activated downstream of the CCR2 receptor (Turner et al., 1998). However, even taking this in to consideration, in our hands we were able to attenuate the C2 α basal activity with both LY294002 and wortmannin quite considerably at the respective concentrations of 30 μ M and 300 nM. C2 β basal activity is largely resistant to LY294002 (even at 30 μ M) but activity is still severely compromised with 300 nM wortmannin (Foster et al., 2003). It would therefore be expected if C2 α/β were involved there would be a detectable inhibitory effect upon T cell chemotaxis at the utilised concentrations of these PI3K inhibitors. The third PI3K isoform C2 γ has an expression profile restricted to the liver, thus is not relevant to this work (Misawa et al., 1998; Ono et al., 1998; Rozycka et al., 1998).

A variety of studies have demonstrated a role for the 3'-phosphoinositide phosphatase PTEN in localising to the trailing and lateral edges of migrating cells with exclusion from the leading edge, a region where PI3K and its products and their effectors (particularly PKB) have been observed to localise (fig. 3.22) (Funamoto et al., 2002; Iijima et al., 2004;

Iijima and Devreotes, 2002; Li et al., 2003b). Over-expression or deficiency of PTEN attenuates or ameliorates leukocyte motility respectively, whilst deficiency of SHIP, another key regulatory phosphatase, has demonstrated to bring about enhanced motility (Fox et al., 2002; Kim et al., 1999a; Tamura et al., 1998). There are however several lines of evidence to suggest that relocalisation of PTEN to the posterior of the cell is not a prerequisite for migration of all cell types. Firstly, the CEM cell line utilised in this study lack PTEN yet the mechanisms of cell motility, with respect to PI3K requirements, appear to be identical to MDC-induced Th2 cell migration, which contain both PTEN and SHIP. Secondly, evidence that suggests perhaps posterior localisation of PTEN may not be an event shared by all migrating cells is substantiated by HL60 cells (a human promyelocytic leukaemia cell) (Xu et al., 2003) and Jurkat cells (Curnock et al., 2003; Sotsios et al., 1999), in which both cell lines lack PTEN and SHIP yet polarise and migrate seemingly normally in response to chemokines. Furthermore, the use of a PKB inhibitor in a CEM cell chemotaxis assay has no effect upon cell migration. Yet the use of the same inhibitor had an effect upon the rate of neutrophil pseudopod extension and migration (Chodniewicz and Zhelev, 2003a), thus further highlighting the potential differences in PI3K requirements between myeloid and lymphoid cells.

Additionally, although the lipid kinase activity of these PI3K isoforms may be severely diminished with the use of PI3K inhibitors, we cannot rule out the possibility that the associated adapter subunits (i.e. p55, p85, p101) are perhaps playing important additional functional roles, participating in protein – protein interactions, distinct from those required for activation of the catalytic subunits. Each of the p85 subunits of the class IA proteins contain two SH2, one SH3 and two proline-rich domains providing a great potential for protein interaction. It has been demonstrated that SDF-1/CXCL12 is able to stimulate the tyrosine phosphorylation of Gab-2 and its co-association with SHP-2 and the p85 adapter subunit. Additionally, using a mutated dominant SHP-2 construct that lacked the phosphatase domain resulted in the attenuation of SDF-1/CXCL12-mediated chemotaxis (Whittaker and Ward, unpublished data). Also, studies have established that SHP-2 can control integrin-mediated cell migration through its interaction with focal adhesion kinase (FAK) (Manes et al., 1999; Oh et al., 1999; Yu et al., 1998a), and SDF-1/CXCL12 is known to tyrosine phosphorylate a number of focal adhesion complex components (Ganju et al., 1998a). Although the principal consequences of these interactions are likely to be a role in

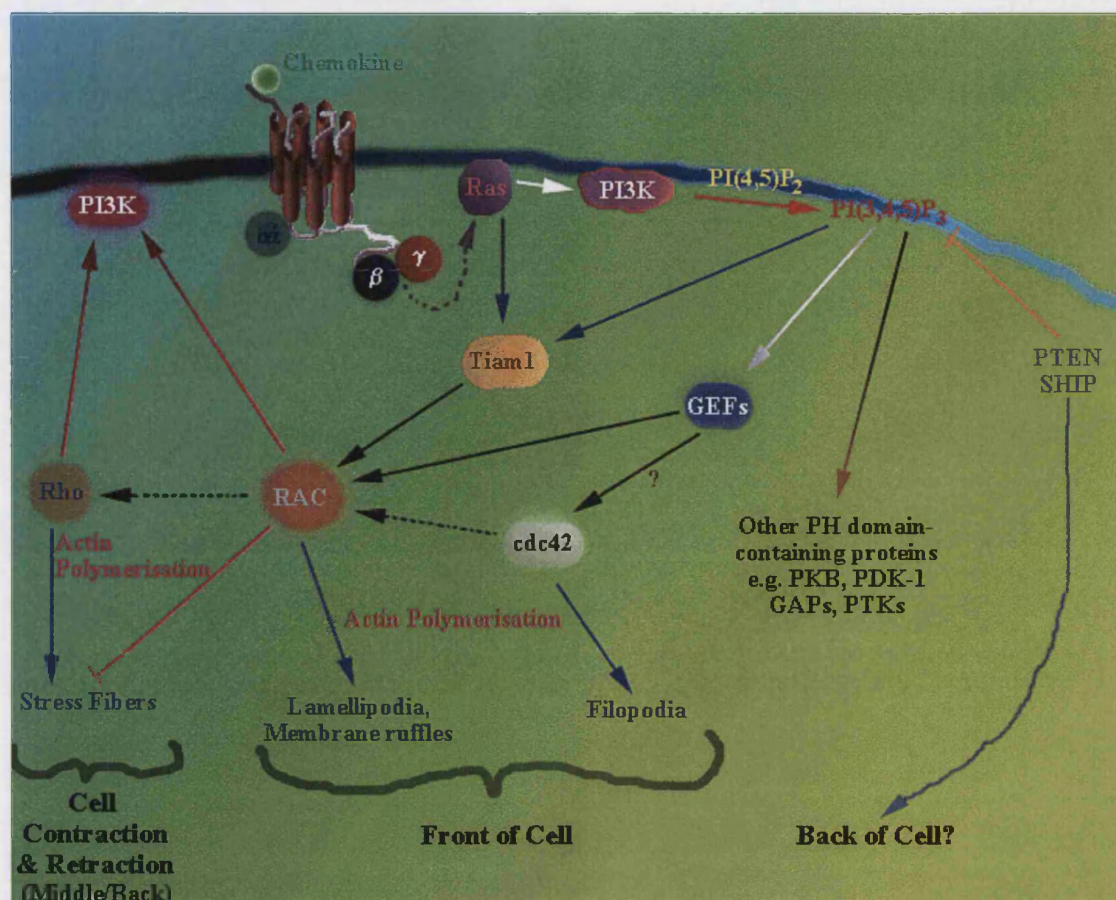


Figure 3.22. Proposed role of PI3K in gradient sensing and subsequent cell migration.

The ligation of the GPCR with chemokine leads to the activation of Ras (or PI3Kγ directly – not depicted) and the subsequent accumulation of PI(3,4,5)P₃ lipids, due to increased PI3K activity. It has been demonstrated in *Dictyostelium* that this accumulation of 3'-phosphoinositides is restricted to the leading edge of the cell due, at least in part, to the localisation of PTEN (and possibly SHIP) to the lateral and trailing edges of the cell. PI(3,4,5)P₃ lipids attract PH domain-containing proteins including guanine-nucleotide exchange factors (GEFs) that activate small GTPases of the Rho family (e.g. Ras, Rac, Rho, cdc42). These Rho GTPases are able to amplify the signal by activating PI3K isoforms themselves. The GTPases activate many different pathways that lead to actin polymerisation (or depolymerisation – not portrayed) and events such as lamellipodia/filopodia formation and cell movement (crawling). *Abbreviations:* GAPs, GTPase activating proteins; PH, pleckstrin homology; PTEN, phosphatase and tensin homology deleted on chromosome ten protein; PTKs, protein tyrosine kinases; SHIP, SH2-containing inositol 5-phosphatase.

the localisation/recruitment/activation of the PI3K catalytic subunits, it cannot not be ruled out that the p85 subunit is recruiting proteins that are having a positive effect upon cell migration without the need of the kinase activity of the associated p110 catalytic subunits

(Ueki et al., 2003). Indeed, it was suggested in a recent study that SHP-2 may dephosphorylate p190 RhoGAP leading to release from lipid rafts and therefore favouring a sustained activation of the small GTPase Rho (Sordella et al., 2003), which may then have an influence on migration.

Possible Alternatives to PI3K-Dependent Pathways in CCR4-Mediated T Cell Migration

The small GTPases Rho, Ras, Rac and cdc42 have been shown to play pivotal roles in the remodelling of the actin cytoskeleton and, consequently, effects on a cells ability to polarise and migrate. Rho-associated coiled-coil forming protein kinase (ROCK), an effector of Rho, enhances myosin light chain (MLC) phosphorylation and thereby regulates actin-myosin contraction (Etienne-Manneville and Hall, 2002), and has been shown to be involved in uropod (posterior of cell) function in migrating neutrophils (Xu et al., 2003). A recent study demonstrated, with the use of a ROCK inhibitor (Y-27632), that CCR7-mediated polarisation and migration of T-lymphocytes requires ROCK (Bardi et al., 2003). This correlates with our observations that inhibition of ROCK also attenuates MDC-mediated T lymphocyte migration (data shown in 'Results II'). Chodniewicz (Chodniewicz and Zhelev, 2003a) demonstrated in neutrophils that 80% of pseudopod extension rate (in response to stimulation with the chemoattractant fMLP) required a PI3K γ -dependent, (similar to levels seen with neutrophil migration from PI3K $\gamma^{-/-}$ mice), pathway and that the remaining 20% was PI3K-independent, with Y-27632 able to inhibit this remaining 20%. This study suggested that in neutrophils it appears that there are two distinct PTX-sensitive pathways, one PI3K γ -dependent and one PI3K-independent. It is possible that T lymphocytes migrating in response to CCR4 ligation (and perhaps CXCR3) do not rely upon a PI3K-dependent pathway but upon at least one or more PI3K-independent pathways, of which one involves Rho-ROCK activity. The possible PI3K-independent pathways will be discussed at greater depth in the 'Discussion II' section.

Additional evidence of cell-specific migration mechanisms occurring within the leukocyte family is highlighted with DOCK2 $^{-/-}$ mice. Upstream of the Rac small GTPases lies the scaffolding protein DOCK2. Lymphocytes from DOCK2 $^{-/-}$ mice revealed a severe lack of a

migratory response to SDF-1/CXCL12, BLC/CXCL13, ELC/CCL19 and SLC/CCL21, yet monocyte/macrophages migrated normally to SDF-1/CXCL12 and MCP-1/CCL2, indicating cell specific mechanisms. It is unclear how DOCK2 is recruited and evidence suggests there could well be PI3K-dependent and independent mechanisms at work (DOCK2 pathways reviewed in (Reif and Cyster, 2002)). Additionally, a recently published paper suggests a reciprocal interaction between Rac and Rho in fibroblasts that is required for cytoskeletal reorganization (Gasteier et al., 2003). More specifically, this study revealed the Rac-dependent activation of the Rho-cascade via a diaphanous-related forming named FHOD1 (formin homology 2 domain containing 1) in inducing cytoskeletal changes. FHOD1 effects required the Rho-ROCK cascade, yet Rac (and not Rho or cdc42) bound to FHOD1 inducing its recruitment to actin filaments and membrane ruffles (Gasteier et al., 2003). This suggests a possible mechanism for the involvement of both a DOCK2 and ROCK-dependent pathway in T lymphocyte directed cell migration without the need of PI3K activity. It could be that there are at least two pathways that are required for T cell chemotaxis, that may or may not be mutually exclusive: one pathway for lamellipodia formation at the anterior of the cell (requiring DOCK2?); and a pathway required for uropod formation at the posterior (ROCK-dependent?). A cell requires the extension at the front to be tightly coordinated temporally with contraction in the middle and retraction at the back for effective movement and prevention of either process will severely affect a cells migration. Consequently, it is more likely that the pathways are intertwined with one another in some way to make this movement finely coordinated, with a negative feedback interaction between these pathways in order to get a coordinated crawling movement of the cell (Meili and Firtel, 2003).

Conclusions I

The data presented in this first results section indicates that CCR4-mediated T-lymphocyte cell migration is independent of detectable PI3K catalytic activity. The ability of MDC to activate PI3K isoforms (class I_A/I_B) suggests a role for them to play in currently non-characterised CCR4-mediated functional responses (such as cell proliferation/anti-apoptosis). Although it appears that neither CEM cells nor polarised Th2 cells require PI3K activity for chemotaxis it would seem that there are some fundamental differences between their responses to the CCR4 ligands. The kinetics for PKB phosphorylation are far more rapid and transient in the non-transformed T cells (and HUT-78 cells), with the transient nature possibly explained by the presence of PTEN. In addition, and perhaps more interestingly of all, TARC and MDC are able, in equal measures, to induce the chemotaxis of Th2 cells, yet TARC is far less effective in instigating CEM cell chemotaxis than MDC. This phenomenon is unexplainable and to date we have been unable to find a difference in the signalling pathways mediated by TARC and MDC in these cells that may provide an explanation. This data suggests, along with the other recent studies mentioned, the involvement of PI3K in chemokine-mediated migration of T lymphocytes is, in all probability, context dependent. Some possible PI3K-independent mechanisms have been discussed but these pathways remain very ambiguous, and will be discussed, along with other possible options, in the remaining part of this thesis.

Key Future Experiments I

1. Perform *in vitro* PI3K assays in Th2 cells – basal PI3K activity would be expected to be lower allowing for better assessment of the extent of their activation.
2. Utilise PI3K isoform knockout models to ascertain the role of PI3K in CCR4-mediated directional T cell migration – currently studies have largely only been performed in granulocytes and not with either of the CCR4 ligands.
3. Use siRNA technology to make certain the role, if any, of PI3K in CCR4-mediated T cell migration – there has recently been an increase in the accessibility of this technology and it offers a number of advantages over current techniques: firstly, it is cheaper and far easier than generating knockout models; secondly, the method allows the control of expression. Thus the cells develop and are cultured with the gene of interest before its ‘knocked out’, allowing for suitable controls, and possibly preventing any abnormalities occurring during development/maturation due to the loss of the target gene.
4. Study the role of the PI3K isoforms and downstream effectors in cell polarisation and migration by the use of real time confocal microscopy – the availability of selective antibodies (raised in different species) against all the PI3K isoforms and many of the effectors would allow the localisation and any mobilisation of these molecules to be viewed, providing strong evidence for the role of PI3K in T cell migration.
5. Attempt to determine why TARC is ineffective in causing the migration of CEM cells yet displays similar potency in attracting human Th2 cells as MDC. This would involve in many types of experiments, including western blot analysis to elucidate any differences in signalling pathways and chemotaxis experiments.
6. Determine the role of PTEN, if any, in T cell migration in response to the CCR4 ligands.

4

RESULTS II

4. RESULTS II

Overview

Having established that the PI3K pathway is activated by both MDC and TARC but is yet surprisingly dispensable for CCR4-mediated T cell migration, investigations were performed to determine the requirement of various other signalling molecules for chemotaxis.

Chemokine receptors, as with all GPCRs, act as ligand-dependent GDP exchange factors with receptor ligation leading to the exchange of GDP for GTP in the receptor-associated GDP-bound G protein. This leads to the G_α and $G_{\beta\gamma}$ subunits dissociating and activating various signalling cascades, with the receptor reverting to a low affinity state (Arai and Charo, 1996; Kuang et al., 1996). A number of studies have demonstrated the activation of a number of components of various signalling pathways for most chemokines and in multiple cell types, including the PI3K and ERK-MAPK pathways (Ashida et al., 2001b; Bonacchi et al., 2001; Chandrasekar et al., 2003a; Smit et al., 2003; Soriano et al., 2003; Sotsios et al., 1999; Wain et al., 2002).

However, thus far there has been very little research on the signalling pathways downstream of CCR4, and this has led us to investigate the CCR4-mediated signal transduction pathways and the cascades that may be involved in T lymphocyte migration. In this study, we demonstrate that ligation of CCR4 leads to activation of the ERK-MAPK pathway, in addition to the PI3K pathway, and that neither of these cascades is required for directed T cell migration. However, phospholipase C (PLC) is required, but interestingly, migration is independent of classical/novel protein kinase C isoforms and calcium mobilisation. The Rho-ROCK pathway also appears to be essential for migration. These alternative PLC- and ROCK-mediated pathways are discussed.

To aid the reader, there will be 'pathway & inhibitor guides' throughout this results section to assist the reader in understanding the pathways being analysed within that current sector.

4.1 Characterisation of CCR4-Mediated Intracellular Calcium Mobilisation

Introduction

Calcium is a ubiquitous intracellular signal that accompanies cells throughout their entire lifespan, from their origin at fertilisation to the end of their life cycle. There has been a rapid advancement of the calcium field since the 1950s, when interest in the signalling role of calcium started. This has led to significant roles for calcium in a plethora of processes covering fertilization, proliferation, apoptosis, contraction and secretion, development, learning and memory (Berridge et al., 2000; Berridge et al., 2003). Calcium, at the cellular level, is derived from two sources – external and internal. It enters from outside the cell by passing through channels that span the plasma membrane, such as voltage-operated calcium channels and receptor-operated calcium channels. Or it can be released from internal calcium stores, through channels in the endoplasmic reticulum (Berridge et al., 1998).

CCR4-Mediated Calcium Mobilisation

All ligated signalling chemokine receptors have demonstrated the intracellular mobilisation of calcium and is thus a widely used indicator of chemokine receptor activation (Jordan et al., 1999; Struyf et al., 2001; Sullivan et al., 1999a; Yoshida et al., 1999). CCR4 has been shown to mobilise calcium in a range of cell types that it is expressed on, including platelets, NK cells and Th2 cells (Abi-Younes et al., 2001; Andrew et al., 1998; Inngjerdingen et al., 2000). Figures 4.1 – 4.4 demonstrate the MDC- and TARC-stimulated intracellular calcium mobilisation in CHOK1hCCR4 cells. This mobilisation is extremely rapid in onset and very transient, with levels returning back to basal within 20 seconds. CHOK1hCCR4 calcium analysis was performed using a microscopy technique that measured emission intensity at λ 510 nm after excitation at both λ 340 nm and λ 380 nm in Fura-2-loaded cells. Upon calcium binding, the fluorescent excitation maximum of the indicator undergoes a blue shift from 363 nm (Calcium-free) to 335 nm (Calcium-saturated), while the fluorescence emission maximum is relatively unchanged at \sim 510 nm.

Graphical analysis of fluorescent intensity at 510 nm for 340 nm and 380 nm will therefore generate 2 graphs that are mirror images of one another (fig. 4.2, *B*, and fig. 4.4, *B*). Results are expressed as the ratio of the fluorescent emission intensity corresponding to the two excitations, with a pseudocolour format utilised for visualisation purposes (fig. 4.2, *A*, and fig. 4.4, *A*). This method allows for the spatial analysis of calcium mobilisation to be determined. Figure 4.5 displays intensity plots at different time points for a single cell stimulated with TARC. Although the resolution is poor, it is reasonably clear that the calcium flux is emanating from the centre of the cell.

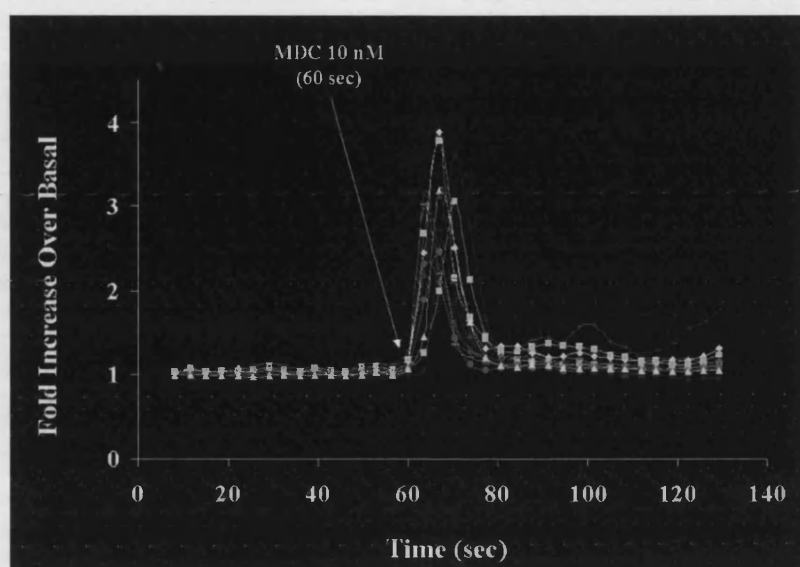
This thesis is focused on CCR4-mediated signalling pathways in T cells. Using another method of calcium flux analysis (FLIPR), which allows for much more of a high-throughput analysis, MDC- and TARC-induced calcium mobilisation in CEM and Th2 cells was measured. As was observed for CHOK1hCCR4 cells, MDC and TARC both elicited rapid intracellular calcium mobilisation in CEM cells (fig. 4.6). However, in contrast, this concentration-dependent mobilisation was much less transient than in CHOK1hCCR4 cells and a return to basal levels was achieved only after at least 90 seconds. It is also apparent that, as is the case for PKB phosphorylation and other signalling/functional responses, MDC is a more potent stimulator of intracellular calcium flux than TARC (fig. 4.7).

Use of Pertussis Toxin to Ascertain the Extent of CCR4-Mediated Calcium Mobilisation Reliance Upon G α i

Chemokines predominantly signal through GPCRs that are coupled to the heterotrimeric G proteins G α i $\beta\gamma$, although other G α proteins have been demonstrated to couple to some chemokine receptors (Arai and Charo, 1996; Damaj et al., 1996; Vila-Coro et al., 1999). Thus, the majority of chemokine-mediated responses are sensitive to the G α i inhibitor pertussis toxin (PTX). PTX catalyses the ADP-ribosylation of G α i, thus preventing the G protein heterotrimers from interacting with receptors. The α i subunits remain in their GDP-bound state, unable to initiate pathway activation. Pretreatment of CEM cells with 100 ng/ml PTX abolished MDC- or TARC-stimulated intracellular calcium flux (fig. 4.8).

Figure 4.1. MDC-mediated calcium mobilisation in CHOK1hCCR4 cells.

A



B

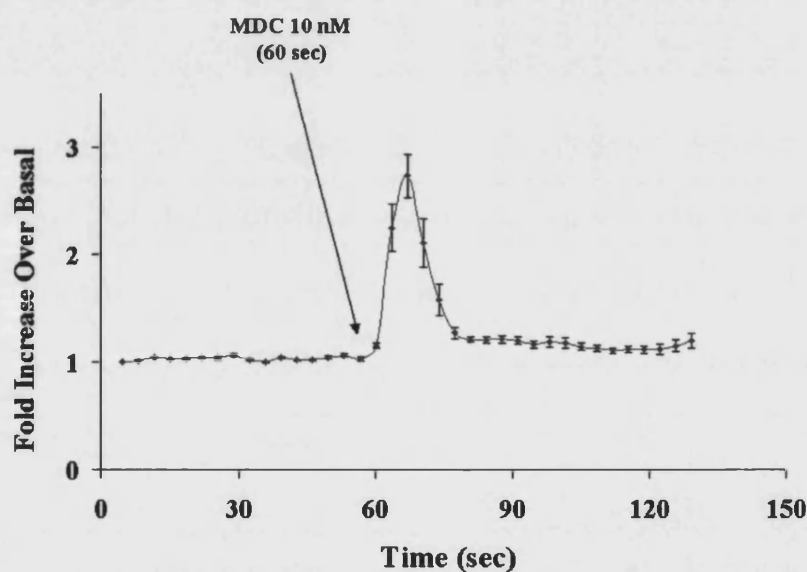


Figure 4.1. MDC-mediated calcium mobilisation in CHOK1hCCR4 cells. Levels of intracellular-free calcium were measured using the MERLIN imaging system and Zeiss Axiovert S100 inverted microscope equipped with an UltraPix FK1000 cooled digital camera and a SpectraMASTER high-speed monochromator. Cells were grown on coverslips to approximately 20% confluency. Prior to experimentation, cells were washed and resuspended in physiological salt solution (PSS) and loaded with Fura-2, as described in *Materials and Methods*. Cells were stimulated with MDC (10 nM) whilst alternately excited with 340 and 380 nm wavelengths. Fura-2 fluorescence emission intensity was measured at λ 510 nm and the 340/380 nm fluorescence ratios (R_f) were calculated for individual cells (A) and the mean calculated (B). Results are expressed as fold increase over basal R_f (\pm SEM, where appropriate), and representative of more than three separate experiments.

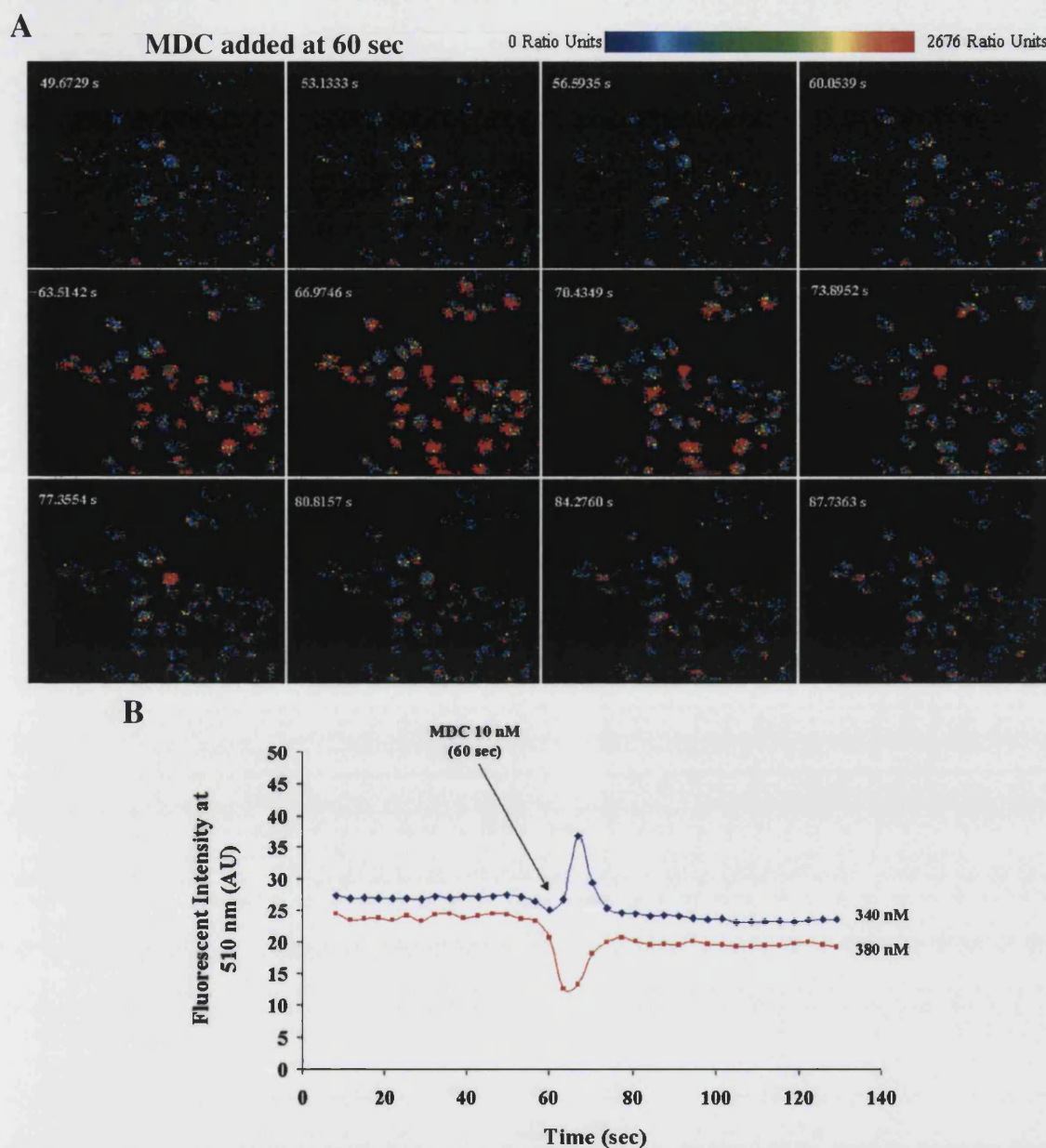
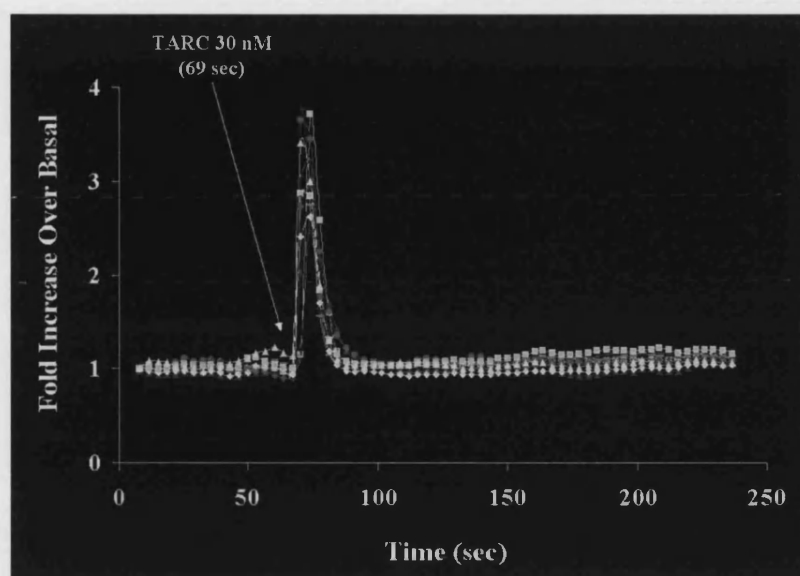
Figure 4.2. Visualisation of MDC-induced calcium mobilisation in CHOK1hCCR4 cells.

Figure 4.2. Visualisation of MDC-mediated calcium mobilisation in CHOK1hCCR4 cells. Levels of intracellular-free calcium were visualised using the MERLIN imaging system and Zeiss Axiovert S100 inverted microscope equipped with an UltraPix FK1000 cooled digital camera and a SpectraMASTER high-speed monochromator. Cells were grown on coverslips to approximately 20% confluency. Prior to experimentation, cells were washed and resuspended in PSS and loaded with Fura-2, as described in *Materials and Methods*. The intensity for fluorescent light emission ($\lambda = 510$ nm) using excitation at 340 and 380 nm was monitored at the level of each single fura-2-loaded cell of the field (A), with MDC (10 nM) added at 60 sec. A representative graph of emission intensity at λ 340 nm and λ 380 nm excitation is presented for a single cell (B). Results are expressed in a pseudocolour format and are indicative of the calculated Rf values for each cell. These results are representative of more than three separate experiments.

Figure 4.3. TARC-mediated calcium mobilisation in CHOK1hCCR4 cells.

A



B

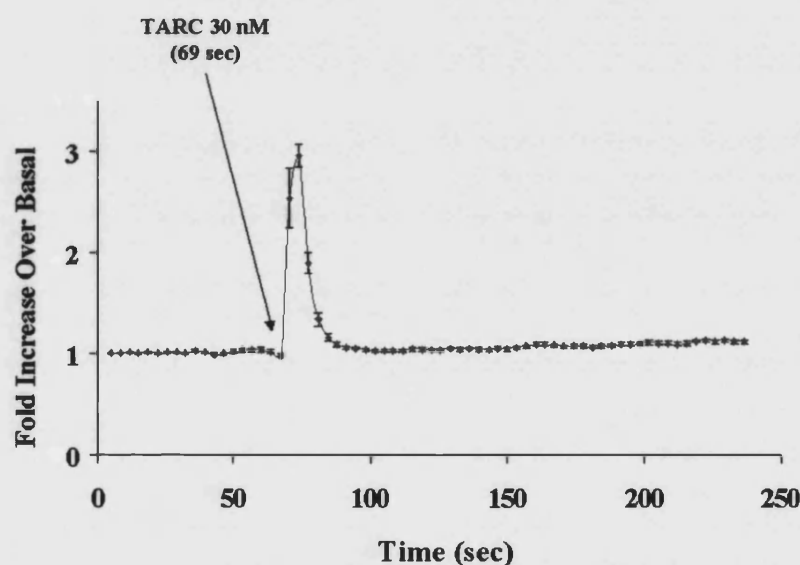


Figure 4.3. TARC-mediated calcium mobilisation in CHOK1hCCR4 cells. Levels of intracellular-free calcium were measured using the MERLIN imaging system and Zeiss Axiovert S100 inverted microscope equipped with an UltraPix FK1000 cooled digital camera and a SpectraMASTER high-speed monochromator. Cells were grown on coverslips to approximately 20% confluency. Prior to experimentation, cells were washed and resuspended in physiological salt solution (PSS) and loaded with Fura-2, as described in *Materials and Methods*. Cells were stimulated with TARC (30 nM) whilst alternately excited with 340 and 380 nm wavelengths. Fura-2 fluorescence emission intensity was measured at λ 510 nm and the 340/380 nm fluorescence ratios (Rf) were calculated for individual cells (A) and the mean calculated (B). Results are expressed as fold increase over basal Rf (\pm SEM, where appropriate), and representative of more than three separate experiments.

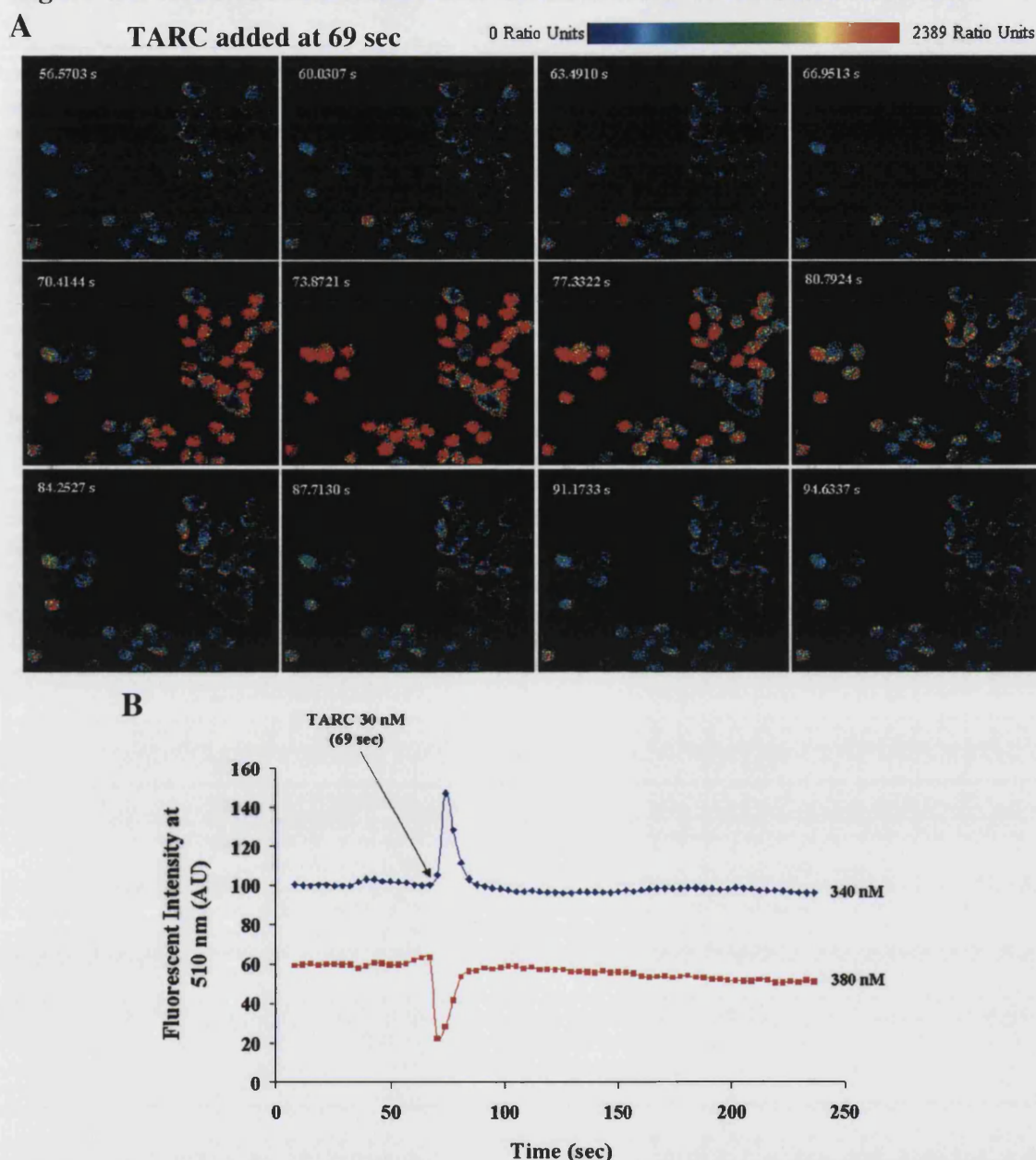
Figure 4.4. Visualisation of TARC-induced calcium flux in CHOK1hCCR4 cells.

Figure 4.4. Visualisation of TARC-induced calcium flux in CHOK1hCCR4 cells. Levels of intracellular-free calcium were visualised using the MERLIN imaging system and Zeiss Axiovert S100 inverted microscope equipped with an UltraPix FK1000 cooled digital camera and a SpectraMASTER high-speed monochromator. Cells were grown on coverslips to approximately 20% confluency. Prior to experimentation, cells were washed and resuspended in PSS and loaded with Fura-2, as described in *Materials and Methods*. The intensity for fluorescent light emission ($\lambda = 510$ nm) using excitation at 340 and 380 nm was monitored at the level of each single fura-2-loaded cell of the field (A), with TARC (10 nM) added at 69 sec. A representative graph of emission intensity at λ 340 nm and λ 380 nm excitation is presented for a single cell (B). Results are expressed in a pseudocolour format and are indicative of the calculated Rf values for each cell. These results are representative of more than three separate experiments.

Figure 4.5. Spatial analysis of TARC-induced calcium mobilisation in CHOK1hCCR4 cells.

TARC added at 69 sec

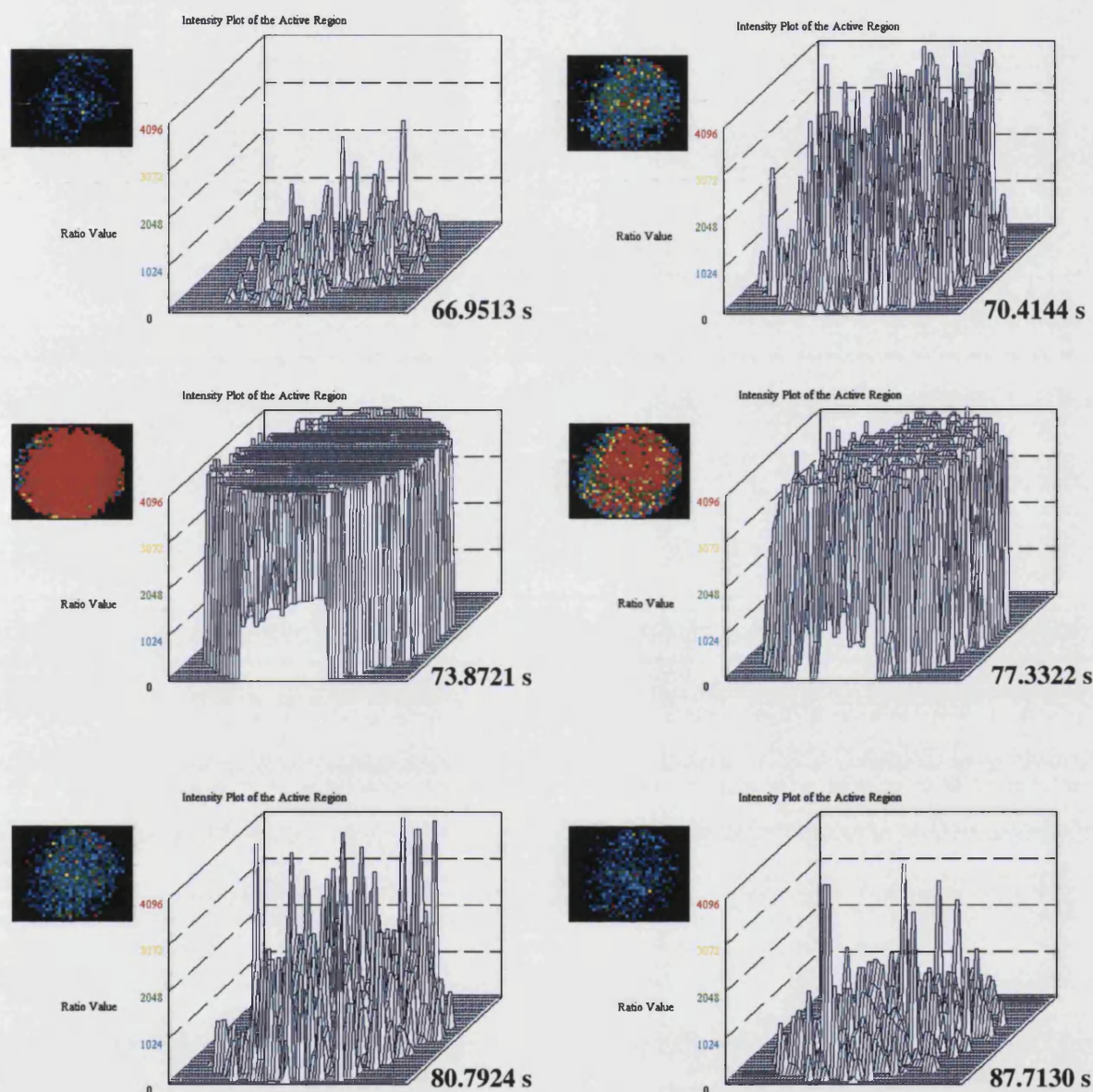
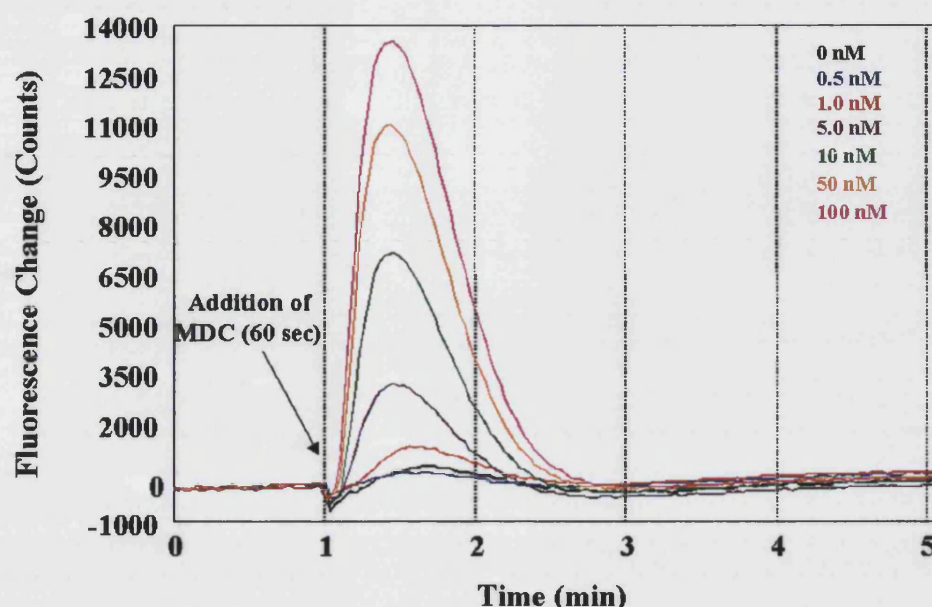


Figure 4.5. Spatial analysis of TARC-induced calcium mobilisation in CHOK1hCCR4 cells. Levels of intracellular-free calcium were visualised using the MERLIN imaging system and Zeiss Axiovert S100 inverted microscope equipped with an UltraPix FK1000 cooled digital camera and a SpectraMASTER high-speed monochromator. Cells were grown on coverslips to approximately 20% confluency. Prior to experimentation, cells were washed and resuspended in PSS and loaded with Fura-2, as described in *Materials and Methods*. The intensity for fluorescent light emission ($\lambda = 510$ nm) using excitation at 340 and 380 nm was monitored at the level of a single fura-2-loaded cell, with TARC (10 nM) added at 69 sec.

Figure 4.6. MDC- and TARC-induced calcium flux in CEM cells is rapid, transient and concentration-dependent, as determined by FLIPR analysis.

A



B

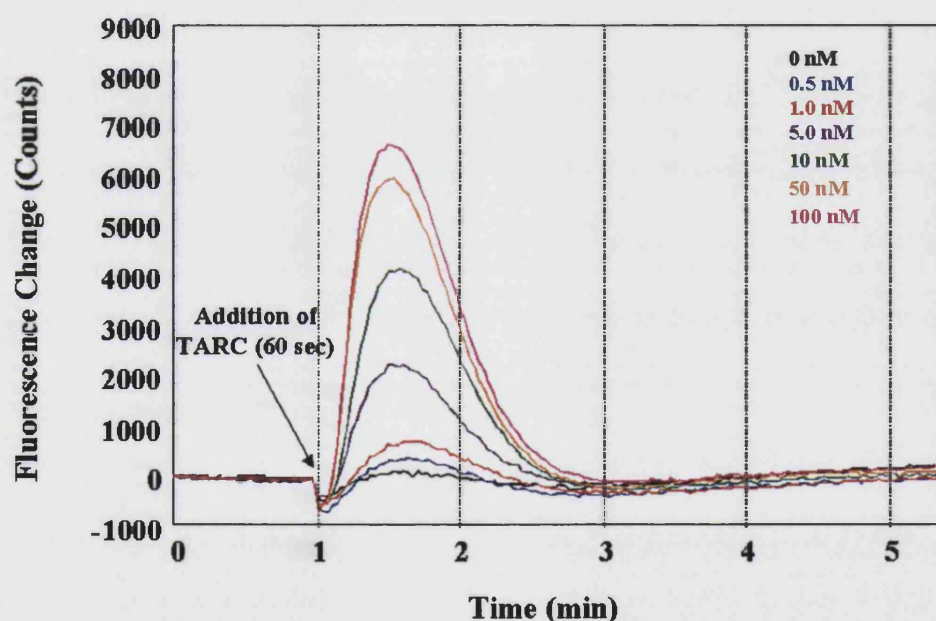


Figure 4.6. MDC- and TARC-induced calcium flux in CEM cells is rapid, transient and concentration-dependent, as determined by FLIPR analysis. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer, labelled with Fluo-4, and plated out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined using a CCD camera with a band-pass interference filter (510–560 nm). Results are representative of more than 3 separate experiments.

Figure 4.7. Comparison of MDC- and TARC-mediated calcium mobilisation in CEM cells.

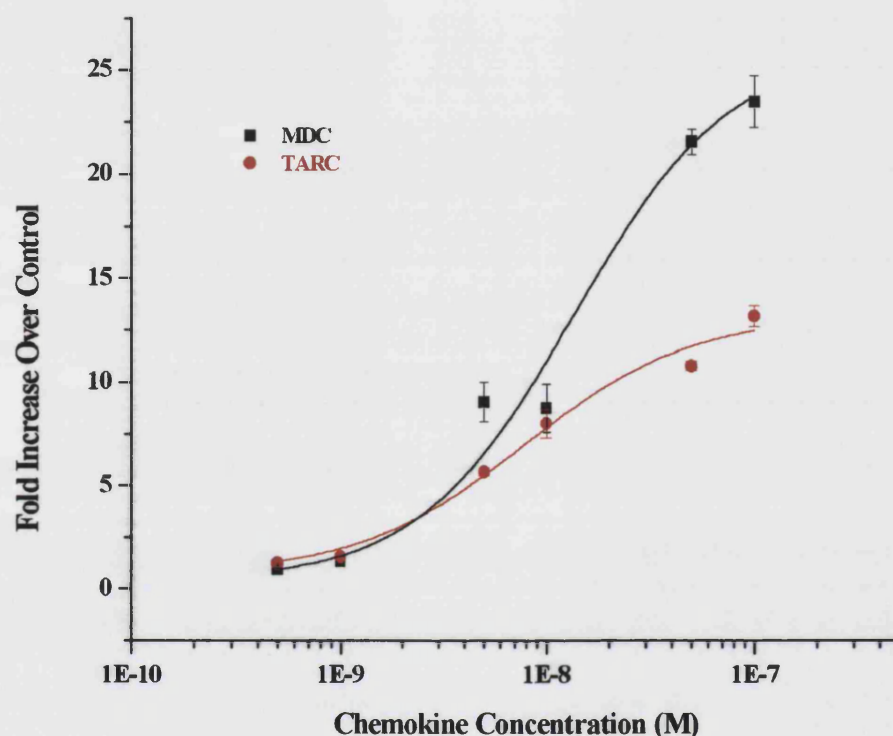


Figure 4.7. Comparison of MDC- and TARC-mediated calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer, labelled with Fluo-4, and aliquoted out at 80, 000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to chemokine stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of more than 3 separate experiments.

Figure 4.8. MDC- and TARC-mediated calcium mobilisation in CEM cells is sensitive to PTX treatment.

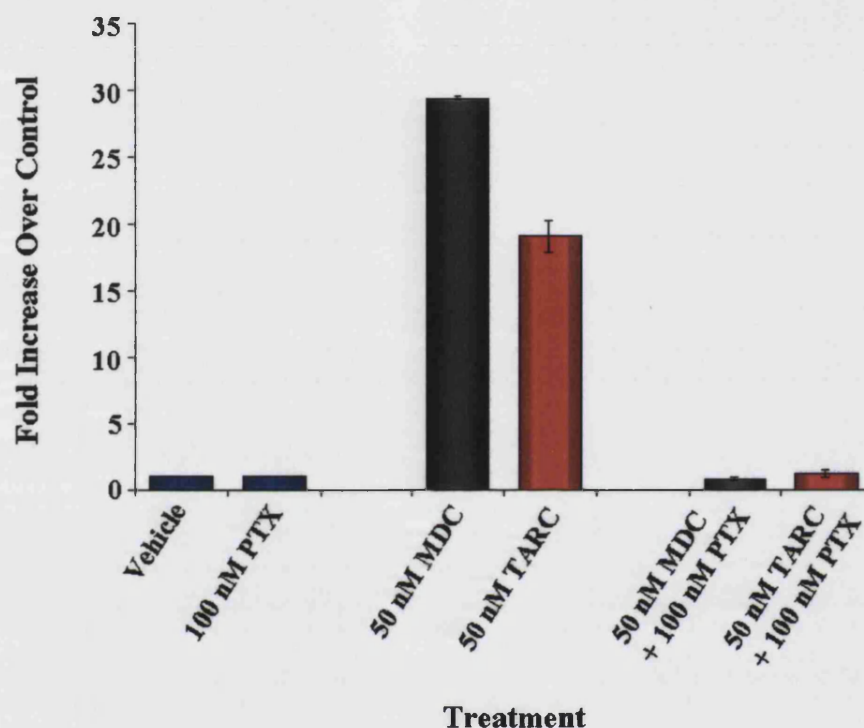
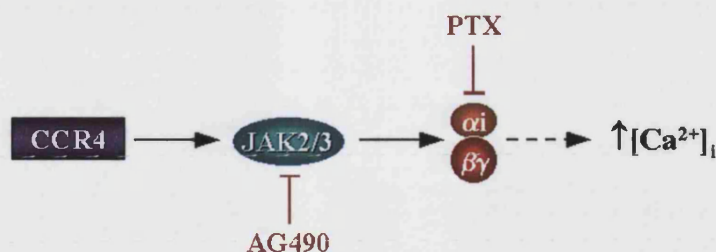


Figure 4.8. MDC- and TARC-mediated calcium mobilisation in CEM cells is sensitive to PTX treatment. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Cells were pretreated with 100 nM pertussis toxin (PTX) overnight (16 hr). Prior to experimentation, cells were washed and resuspended in loading buffer, labelled with Fluo-4, and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to chemokine stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle) or PTX-treated cells (\pm SEM), as appropriate. Results are representative of 3 separate experiments.

Requirement of Janus Kinases for CCR4-Mediated Calcium Mobilisation in CEM Cells



The janus kinase (JAK) family of tyrosine kinases is well known to be activated by many cytokines and growth factors. These JAKs are activated directly by the activated receptors and go on to phosphorylate and activate signal transducers and activators of transcription (STATs). These receptor-associated STATs then dimerise and translocate to the nucleus and regulate gene expression. In the last few years a number of papers have demonstrated that chemokine receptor dimerisation leads to the activation of the JAK-STAT pathway (Mellado et al., 2001; Rodriguez-Frade et al., 1999b; Rodriguez-Frade et al., 1999a; Vila-Coro et al., 1999; Wong et al., 1997; Wong et al., 2001). JAK2/3 activation has been demonstrated to be required for chemokine-mediated induction of intracellular calcium mobilisation in a variety of cell types, including T cells (Mellado et al., 1998; Soriano et al., 2003). To determine if there was any involvement of JAK2/3 in CCR4-mediated calcium flux in T cells, the widely used JAK2/3 inhibitor AG490 was preincubated with CEM cells, with a range of concentrations, for one hour. There was little effect of AG490 at 10 μM and 50 μM compared to MDC-only stimulated calcium mobilisation responses, especially at the lower chemokine concentrations (fig. 4.9). However, preincubation of CEM cells with 100 μM AG490 attenuated the MDC-mediated calcium flux by around 50% at 100 nM MDC. Intracellular calcium mobilisation mediated by TARC displayed similar patterns to that observed for MDC (fig. 4.10). Both JAK2 and JAK3 have been characterised downstream of activated chemokine receptors such as CXCR4 and CCR5 (Vila-Coro et al., 1999; Wong et al., 2001; Zhang et al., 2001). AG490 is known to demonstrate inhibition of both JAK2 and JAK3. In order to elucidate if both JAK2 and JAK3 are having an effect on calcium mobilisation or if it is just one of them, a JAK3 inhibitor that has no effect on JAK2 activity was used. Pretreatment of CEM cells with the JAK3 inhibitor WHI-P154 had no effect on MDC-induced calcium responses and demonstrated an exact profile to that observed with the negative control for AG490, AG9 (an inactive tyrphostin) (fig. 4.11).

Figure 4.9. The JAK2 tyrosine kinase is not essential for an intracellular calcium flux in CEM cells in response to CCR4 ligation with MDC.

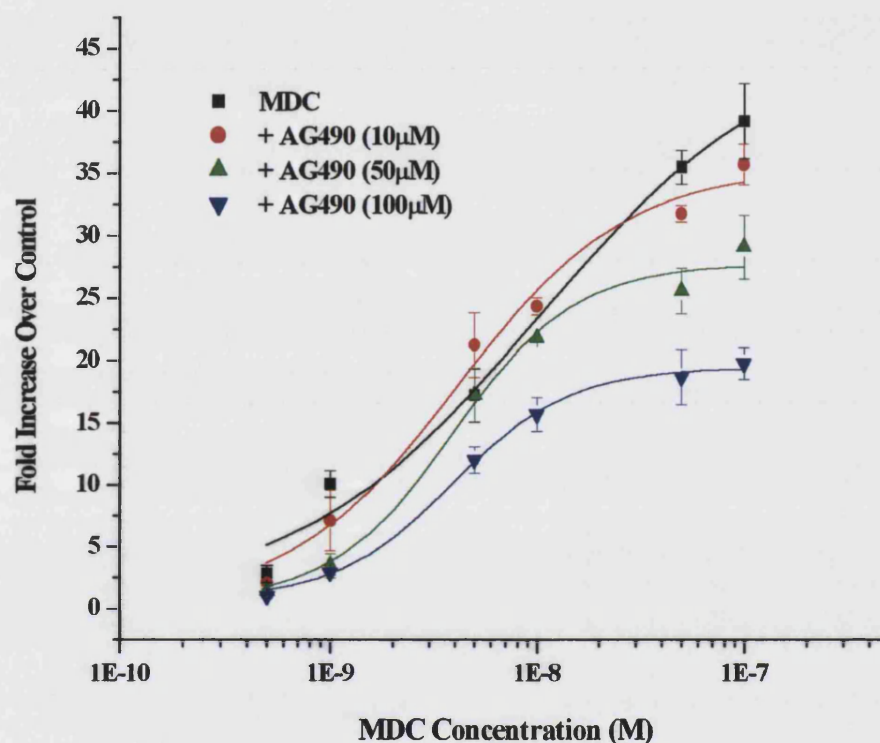


Figure 4.9. The JAK2 tyrosine kinase is not essential for an intracellular calcium flux in CEM cells in response to CCR4 ligation with MDC. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with AG490 for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.10. The JAK2 tyrosine kinase is not essential for an intracellular calcium flux in CEM cells in response to CCR4 ligation with TARC.

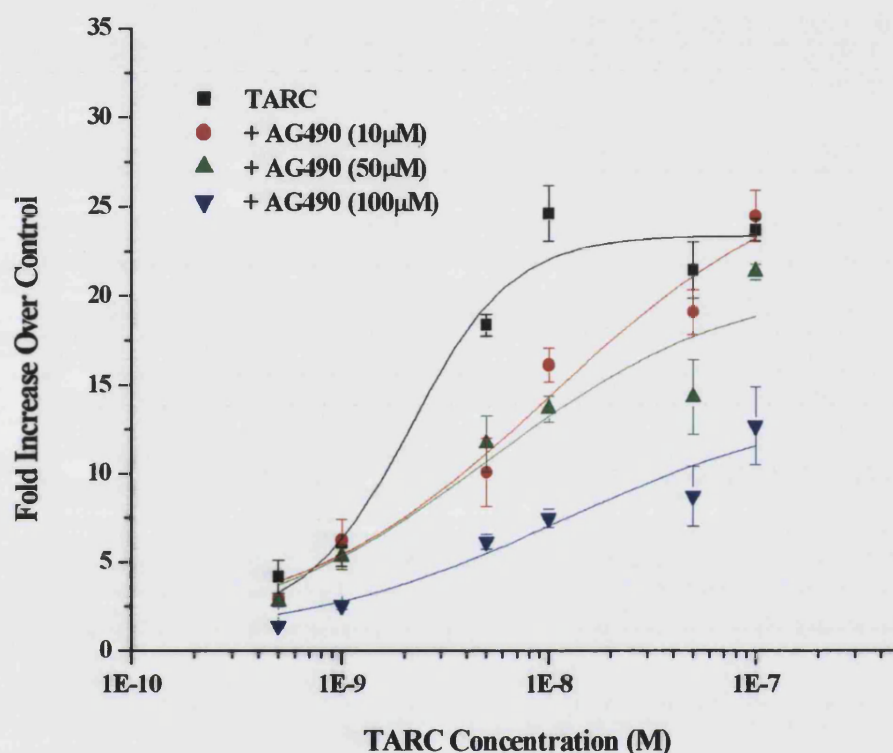


Figure 4.10. The JAK2 tyrosine kinase is not essential for an intracellular calcium flux in CEM cells in response to CCR4 ligation with TARC. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with AG490 for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to TARC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.11. CCR4-mediated intracellular calcium mobilisation does not rely upon JAK3 in CEM cells.

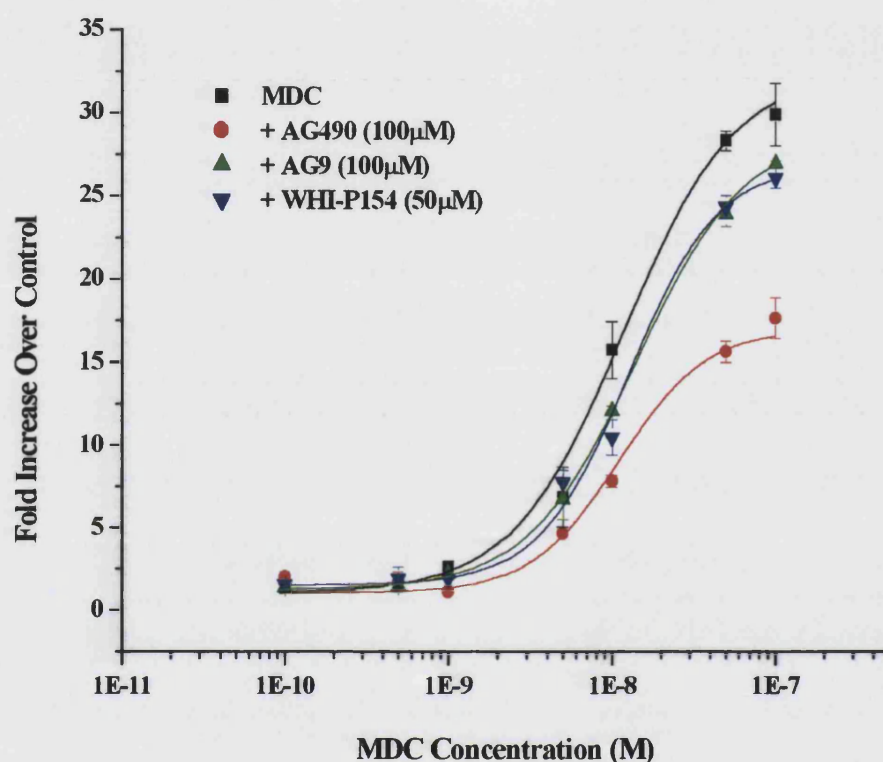
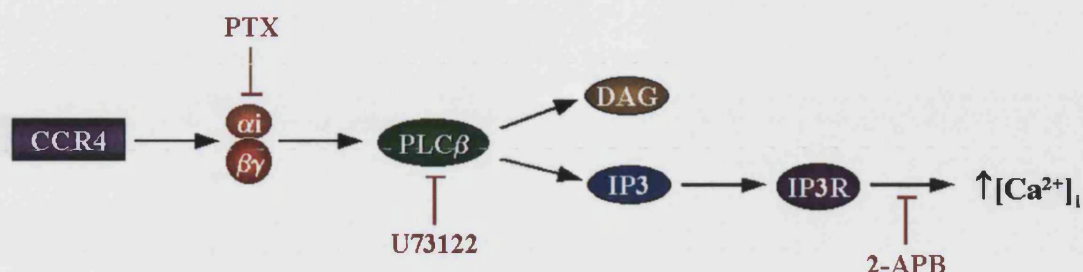


Figure 4.11. CCR4-mediated intracellular calcium mobilisation does not rely upon JAK3 in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with specified inhibitors (WHI-P154 = JAK3; AG9 = negative control for AG490) for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 2 separate experiments.

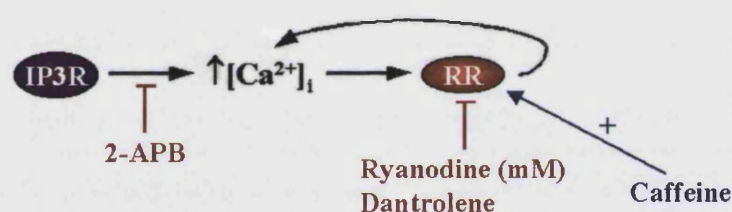
CCR4-Stimulated Calcium Mobilisation is PLC-Dependent and Uses IP3 Receptor Calcium Stores



Calcium releases from the internal stores of the ER are controlled by a variety of channels that include the IP3 receptor (IP3R), ryanodine receptor (RR) and nicotinic acid adenine dinucleotide phosphate receptor (NAADPR) (Bootman et al., 2001; Genazzani and Thorn, 2002; Rizzuto et al., 2002). The most intensely studied of these channels is that of the IP3R. IP3, along with DAG, is a product of the hydrolysis of the membrane phospholipid PI(4,5)P₂ by the lipid enzyme PLC. PLC β can be activated by the G $\beta\gamma$ subunits after chemokine receptor ligation (Jiang et al., 1997; Li et al., 2000; Wu et al., 1992). To determine whether the increase in intracellular calcium following ligation of the CCR4 receptor requires the enzymatic function of PLC isoforms, experiments utilised the pharmacological inhibitor of PLC, U73122 (not specific for any one isoform). The MDC-induced calcium response was concentration-dependently curtailed following an hour pretreatment of CEM cells with U73122 (fig. 4.12). At the highest concentration of inhibitor (1.5 μM) the calcium flux was abolished. As would be expected, TARC-mediated calcium mobilisation demonstrated a similar sensitivity to U73122 as to that observed for MDC (fig. 4.13). The results presented here support the observation of previous studies that have reported U73122 to have an IC₅₀ between 500 nM and 2.1 μM (Smith et al., 1996; Stam et al., 1998; Yule and Williams, 1992). U73343 is the inactive analogue of U73122 and consequently has no effect upon CCR4-induced intracellular calcium mobilisation (fig. 4.14, A and B).

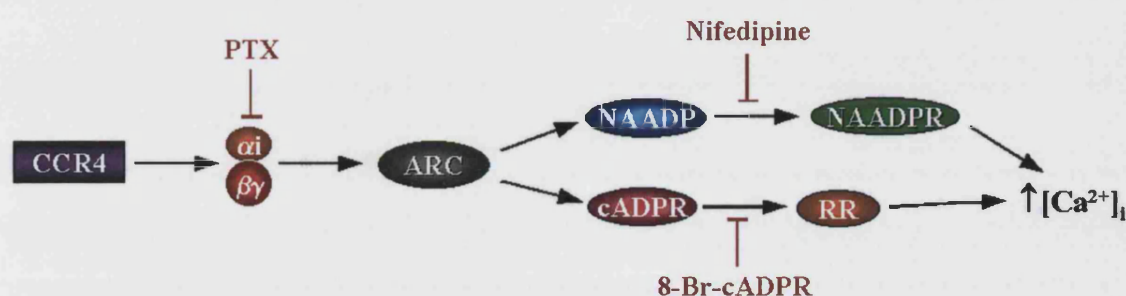
Having determined that PLC is required for calcium mobilisation, it was then necessary to confirm that IP₃ was the mediator of this response. 2-aminoethoxydiphenyl borate (2-APB) has been described as an inhibitor of calcium release from IP₃R stores without affecting binding of IP₃ to IP₃R. It has no effect on calcium release from RR stores and has an IC₅₀ of 42 μ M, with studies using the compound at up to 100 μ M (Ascher-Landsberg et al., 1999; Maruyama et al., 1997; Werry et al., 2003). Treatment of CEM cells with 75 μ M 2-APB led to the abrogation of both MDC- (fig. 4.15, A) and TARC- (fig. 4.15, B) induced intracellular calcium mobilisation.

Ryanodine Receptor Stores do not Contribute Towards CCR4-Mediated Intracellular Calcium Mobilisation in CEM Cells.



The RR calcium stores are released by calcium itself that has been itself released from other sources, normally the IP₃R stores (Bootman et al., 2001; Islam, 2002). Therefore, to test whether 2-APB is just inhibiting the release of calcium from the upper most channel (i.e. IP₃R) in a cascade or that IP₃R stores are the only calcium stores contributing to the CCR4-mediated calcium response, it is necessary to inhibit the RRs and other calcium induced calcium release (CICR) channels. The RRs are termed because ryanodine is a high affinity ligand for the CICR channel and will promote the channel's activation at low concentrations. However, at high concentrations (100 μ M – 10 mM) ryanodine inhibits the channel whilst in its open state (Ehrlich et al., 1994). In addition, at millimolar concentrations, caffeine is an activator of RRs (Friel and Tsien, 1992). Therefore, the combined treatment of caffeine (to open the RR channels) and ryanodine (to block) is useful pharmacological tool for determining the contribution of the ryanodine channel to the calcium response.

MDC- and TARC-induced calcium release from intracellular stores is substantially potentiated in the presence of 10 mM caffeine, but caffeine has no effect on its own in CEM cells (fig. 4.16). Ryanodine has no effect upon CCR4-mediated calcium responses alone but does abolish the effect of caffeine on CCR4-mediated intracellular calcium release (fig. 4.16). The cell permeable RR blocker dantrolene has no effect on either MDC- or TARC-induced calcium release at 100 μ M (fig. 4.17, A and B).



Cyclic adenosine diphosphate-ribose (cADPR) is a second messenger, generated from its precursor NAD⁺ (nicotinamide adenine dinucleotide) possibly by receptor-activated ADP-ribosyl cyclases (ARC), which can gate the RRs and cause the release of calcium (Galione and Churchill, 2000). A metabolite of an alternative pyridine nucleotide, nicotinamide adenine dinucleotide diphosphate (NADP⁺), by possibly a similar process is nicotinic acid adenine dinucleotide phosphate (NAADP). NAADP is able to mobilise intracellular calcium stores distinct from those for IP₃R and RR (Genazzani and Billington, 2002; Patel et al., 2001). Mobilisation of intracellular calcium in NK cells through the cADPR has been demonstrated for IL-8, SDF-1, RANTES and MDC, with DCs mobilising calcium in a cADPR-dependent manner in response to CCL2, CCL19, CCL21 and CXCL12 (Inngjerdingen et al., 1999; Partida-Sanchez et al., 2004). 8-Br-cADPR is an effective inhibitor of cADPR-mediated calcium release (with no effect on ryanodine-mediated calcium release) but had no impact on either MDC- or TARC-induced intracellular calcium release in CEM cells (fig. 4.18 and fig 4.19) (Guse et al., 1995). Nifedipine is an L-type calcium channel blocker but has been shown at very high concentrations to inhibit NAADP-mediated calcium release from intracellular stores, with no effect on IP₃- or cADPR-mediated release (Yusufi et al., 2002). 50 μ M nifedipine pretreatment of CEM cells had a small negative effect upon MDC- and TARC-induced calcium responses at 50 nM and 100 nM chemokine concentrations (fig. 4.18 and fig. 4.19).

Role of PI3K in CCR4-Mediated Calcium Responses

PI3K has been shown to be required for the initiation of calcium entry through calcium-permeable non-selective cation channel 2 (NSCC-2) and store-operated calcium channels (SOCCs) (Kawanabe et al., 2002; Kawanabe et al., 2003). However, using LY294002 to inhibit PI3K activity, there was no effect on MDC- or TARC-induced calcium responses in CEM cells, even at inhibitor concentrations as high as 50 μ M (fig. 4.20 and fig. 4.21).

PKC Sensitive Calcium Mobilisation in Response to CCR4 Ligation

PKC has been demonstrated to have effects on chemokine-mediated calcium mobilisation due to effects on receptor internalisation. Activating PKC has been shown to abrogate chemokine-mediated calcium responses due to receptor internalisation (Guinamard et al., 1999; Schondorf et al., 1993). Figure 4.22, A and B, illustrate that PKC activation, with PMA, abrogated calcium mobilisation. An increase in calcium flux was seen when PKC was inhibited with the broad-spectrum (classical and novel isoform) PKC inhibitor RO-32-0432, or when PKC expression was down-regulated by overnight pretreatment with PMA (fig. 4.22).

CCR4-Mediated Calcium Responses in Th2 Cells

To determine whether CEM cells act in a comparable manner to more physiologically normal CCR4 expressing T cells, *in vitro* generated Th2 cells were analysed for their calcium responses upon CCR4 ligation. MDC and TARC were both able to elicit intracellular calcium mobilisation in Th2 cells and these responses showed similar sensitivities to the PLC and JAK2/3 inhibitors, U73122 and AG490, to those observed for CCR4-mediated calcium responses in CEM cells. U73122 abrogated the calcium responses to MDC and TARC, whereas AG490 had a diminutive effect (fig. 4.23 and fig. 4.24).

Figure 4.12. Inhibition of PLC attenuates the MDC-induced calcium mobilisation in CEM cells.

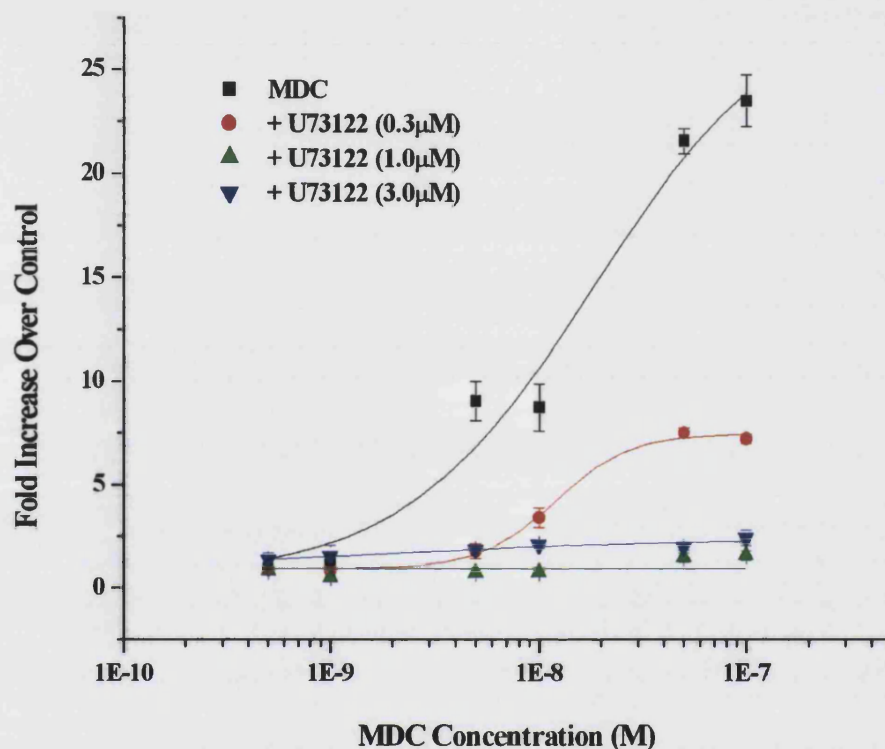


Figure 4.12. Inhibition of PLC attenuates the MDC-induced calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with U73122 for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80, 000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 at least separate experiments.

Figure 4.13. Inhibition of PLC attenuates the TARC-induced calcium mobilisation in CEM cells.

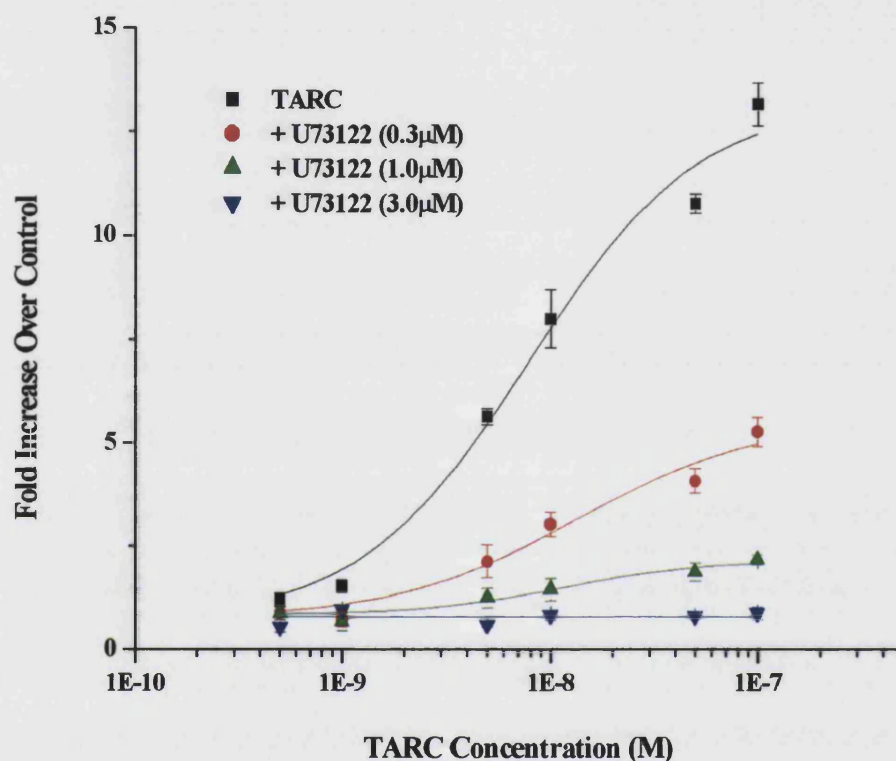


Figure 4.13. Inhibition of PLC attenuates the TARC-induced calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with U73122 for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80, 000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to TARC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of at least 3 separate experiments.

Figure 4.14. The inactive analogue of U73122 has no effect on CCR4-mediated calcium mobilisation in CEM cells.

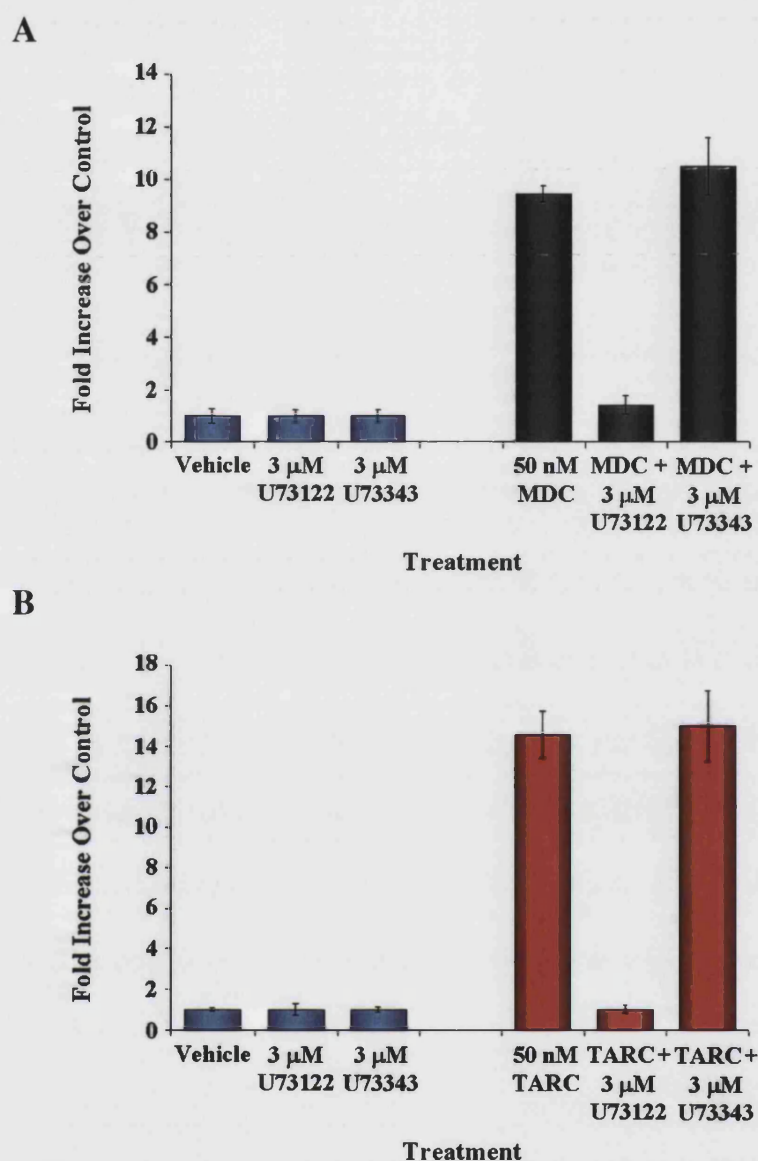


Figure 4.14. The inactive analogue of U73122 has no effect on CCR4-mediated calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with U73122 or U73343 for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC (A) or TARC (B) stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM). Results are representative of 3 separate experiments.

Figure 4.15. CCR4-induced intracellular calcium flux requires calcium release from IP3 receptor stores.

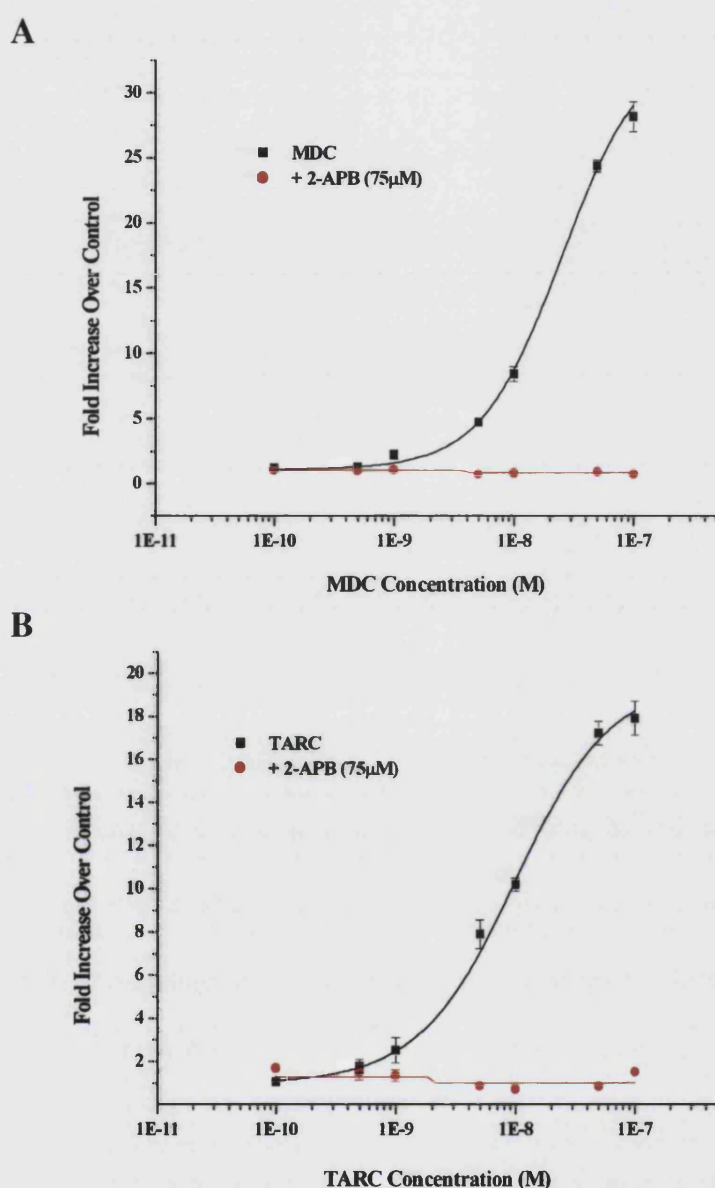


Figure 4.15. CCR4-induced intracellular calcium flux requires calcium release from IP3 receptor stores. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with 2-APB (75 μM) for 1 hr. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC (A) or TARC (B) stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of at least 3 separate experiments.

Figure 4.16. Caffeine pre-treatment potentiates intracellular calcium mobilisation upon CCR4-ligation on CEM cells.

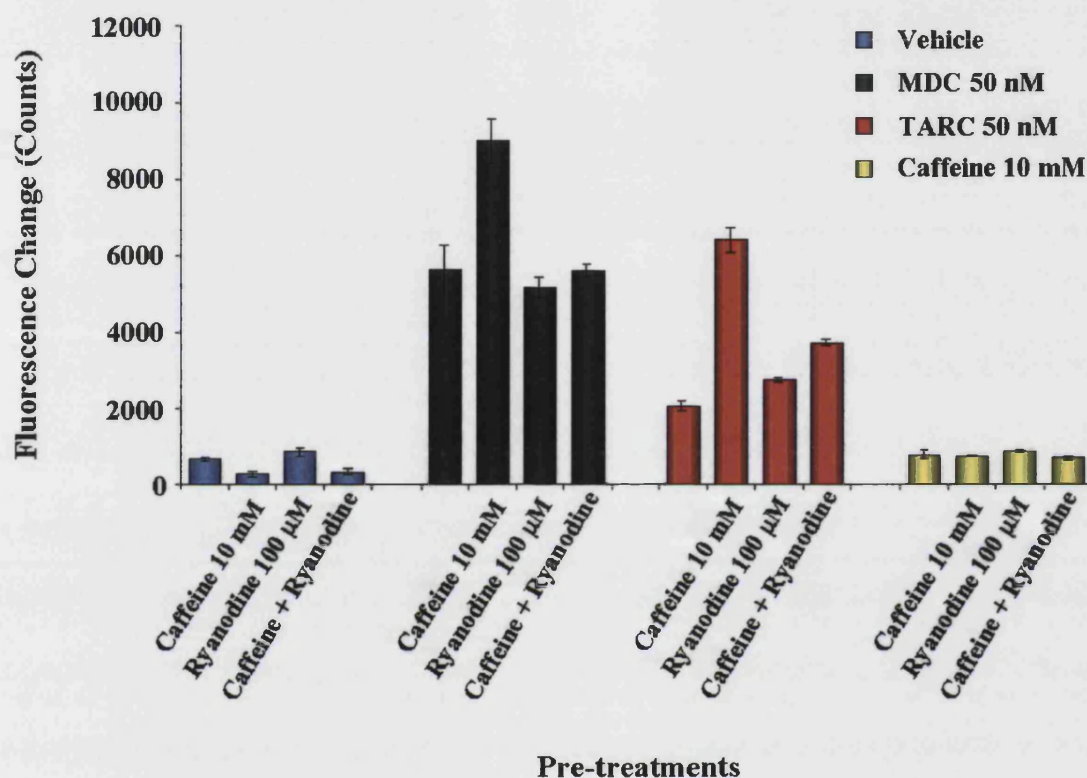


Figure 4.16. Caffeine pre-treatment potentiates intracellular calcium mobilisation upon CCR4-ligation on CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with caffeine, ryanodine or both for 1 hr at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC, TARC or caffeine stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as change in fluorescence (\pm SEM). Results are representative of 3 separate experiments.

Figure 4.17. The ryanodine receptor inhibitor dantrolene has no negative effect upon CCR4-mediated intracellular calcium mobilisation in CEM cells.

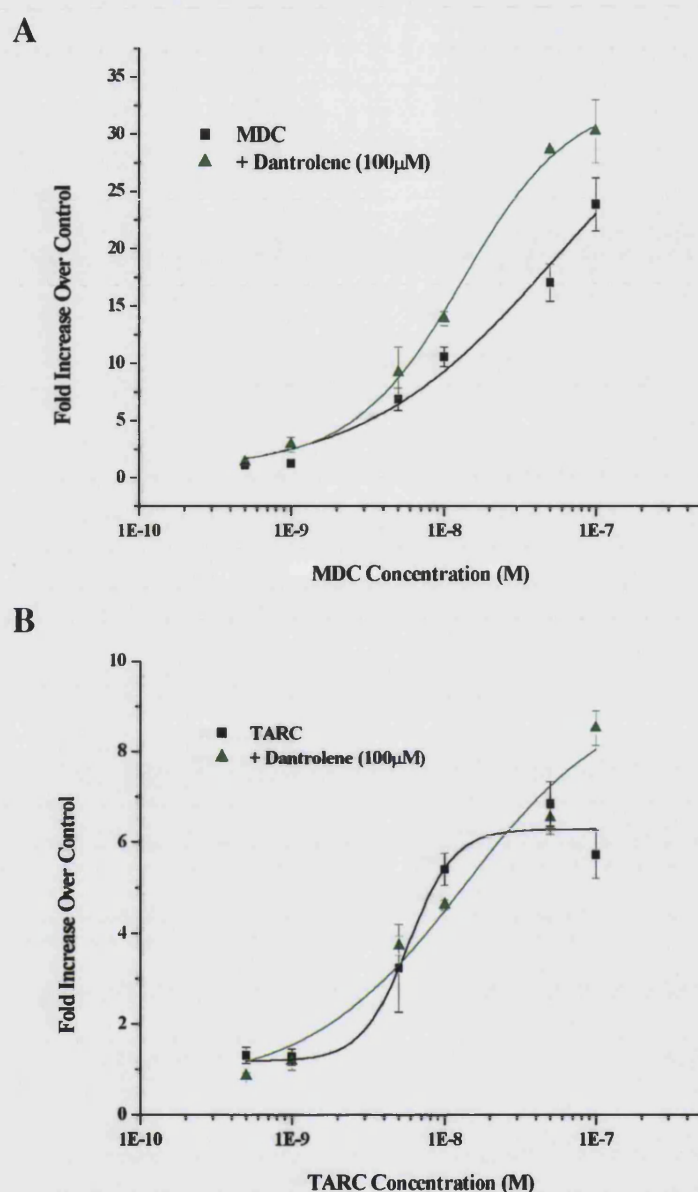


Figure 4.17. The ryanodine receptor inhibitor dantrolene has no negative effect upon CCR4-mediated intracellular calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with dantrolene (100 μ M) for 1 hr. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC (A) or TARC (B) stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 2 separate experiments.

Figure 4.18. cADPR calcium stores play no major contribution towards MDC-induced cytosolic calcium increases in CEM cells.

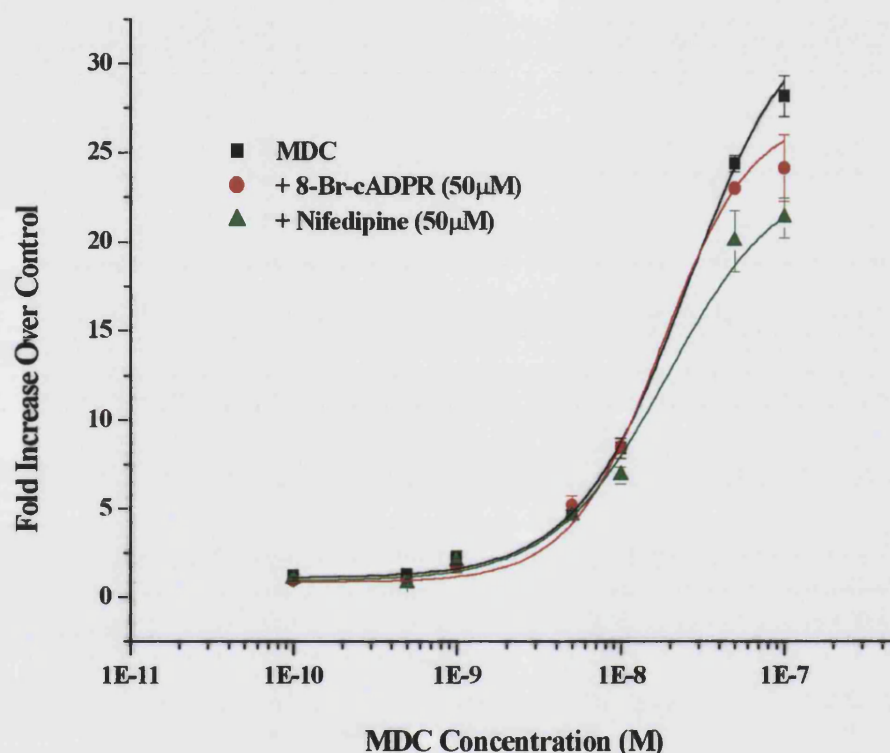


Figure 4.18. cADPR calcium stores play no major contribution towards MDC-induced cytosolic calcium increases in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with 8-Br-cADPR (50 μ M) or nifedipine (50 μ M) for 1 hr. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.19. cADPR calcium stores play no major contribution towards TARC-induced cytosolic calcium increases in CEM cells.

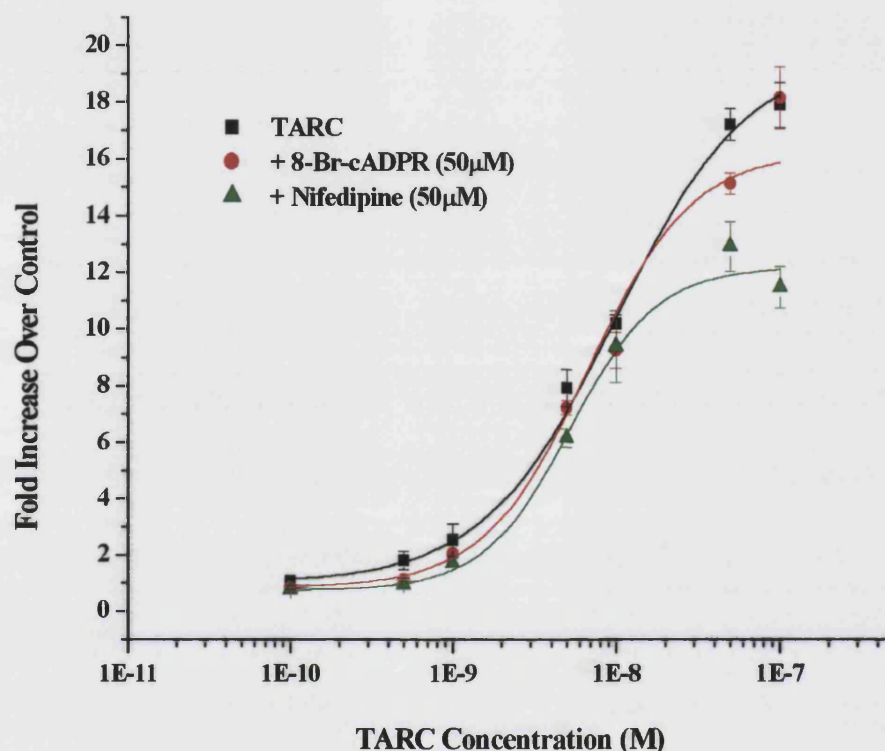


Figure 4.19. cADPR calcium stores play no major contribution towards TARC-induced cytosolic calcium increases in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with 8-Br-cADPR (50 μM) or nifedipine (50 μM) for 1 hr. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to TARC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.20. PI3K inhibition has no effect on MDC-induced intracellular calcium mobilisation in CEM cells.

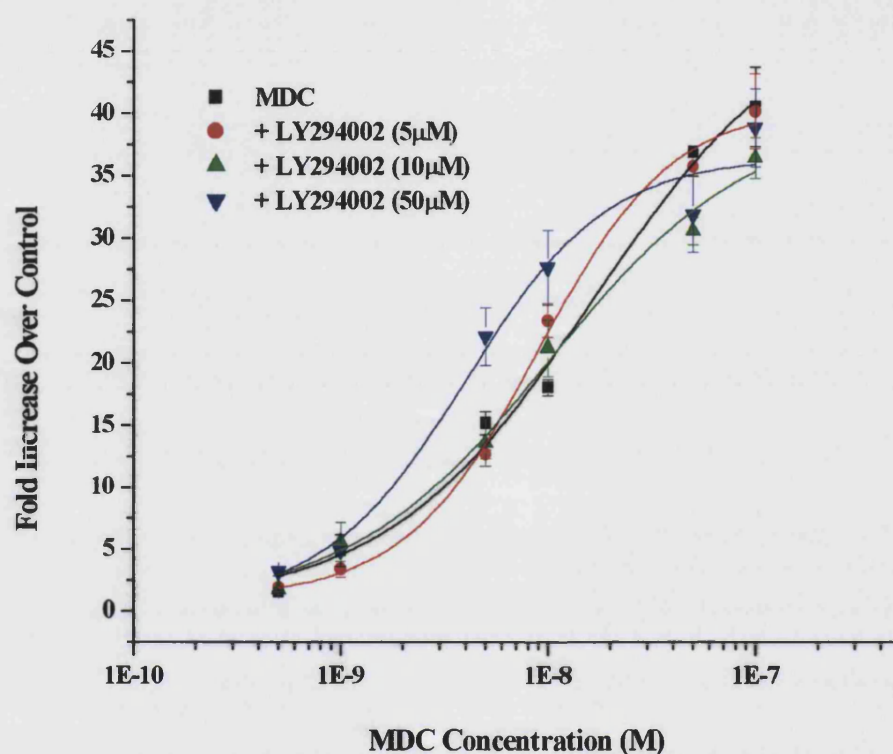


Figure 4.20. PI3K inhibition has no effect on MDC-induced intracellular calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with LY294002 for 30 min at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.21. PI3K inhibition has no effect on TARC-induced intracellular calcium mobilisation in CEM cells.

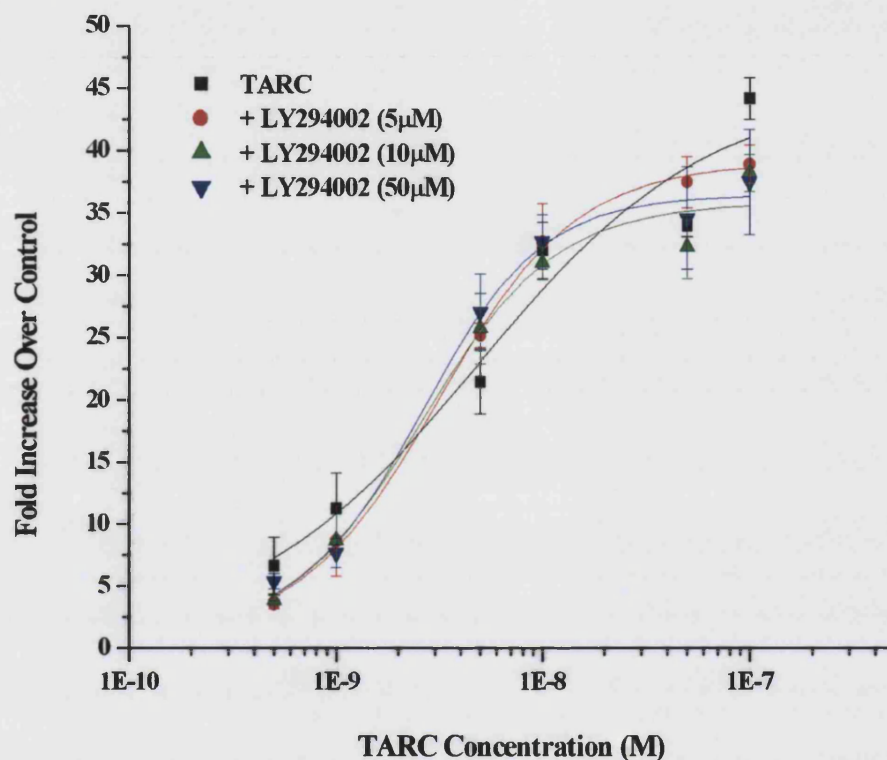


Figure 4.21. PI3K inhibition has no effect on TARC-induced intracellular calcium mobilisation in CEM cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with LY294002 for 30 min at indicated concentrations. Cells were then labelled with Fluo-4 and aliquoted out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to TARC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.22. Attenuation of CCR4-mediated intracellular calcium mobilisation can be achieved through activation of PKC.

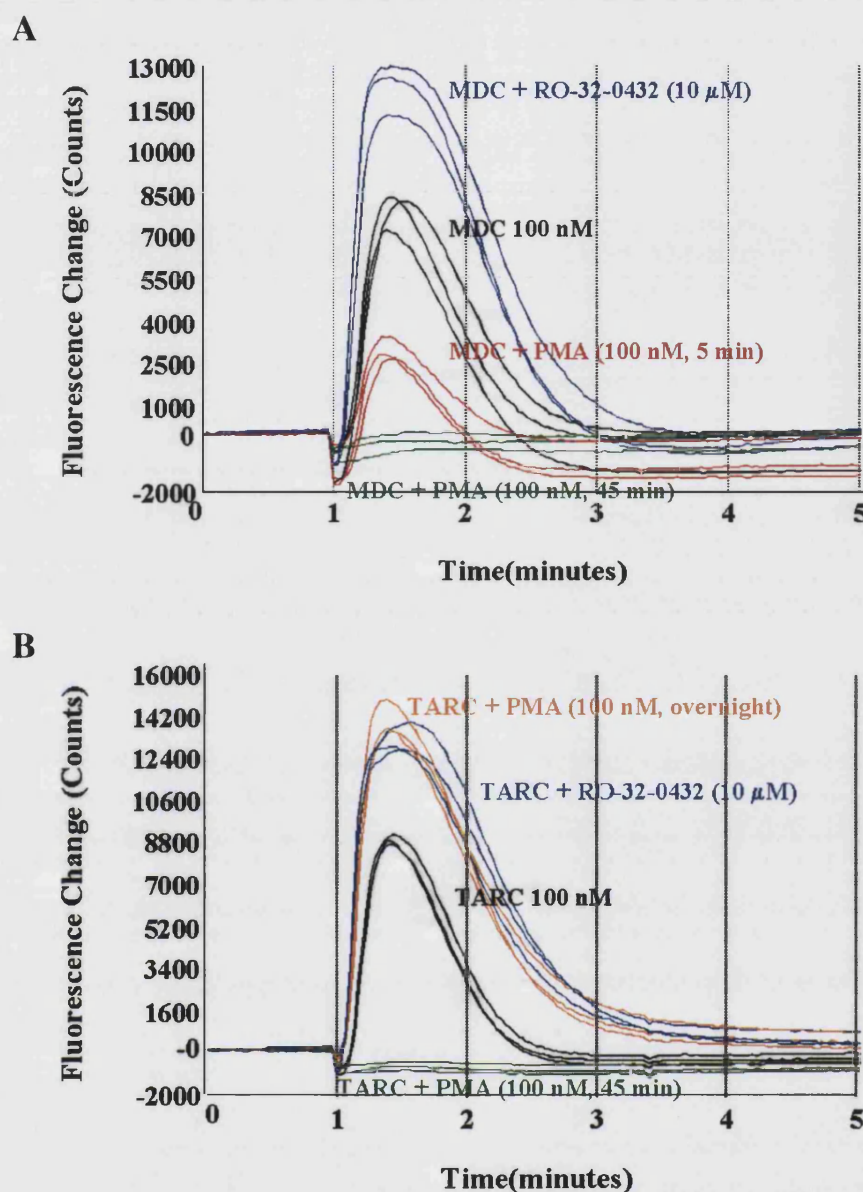


Figure 4.22. Attenuation of CCR4-mediated intracellular calcium mobilisation can be achieved through activation of PKC. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. A sample of cells were treated with 100 nM PMA overnight. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with RO-32-0432 (10 μ M) or PMA (100 nM) for 30 min, as indicated. Cells were labelled with Fluo-4 and plated out at 80,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC (A) or TARC (B) stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). A selection of cells were stimulated with 100 nM PMA for 5 min prior to the addition of MDC. Results are representative of at least 2 separate experiments.

Figure 4.23. Intracellular calcium mobilisation, mediated by MDC, requires PLC activation in Th2 cells.

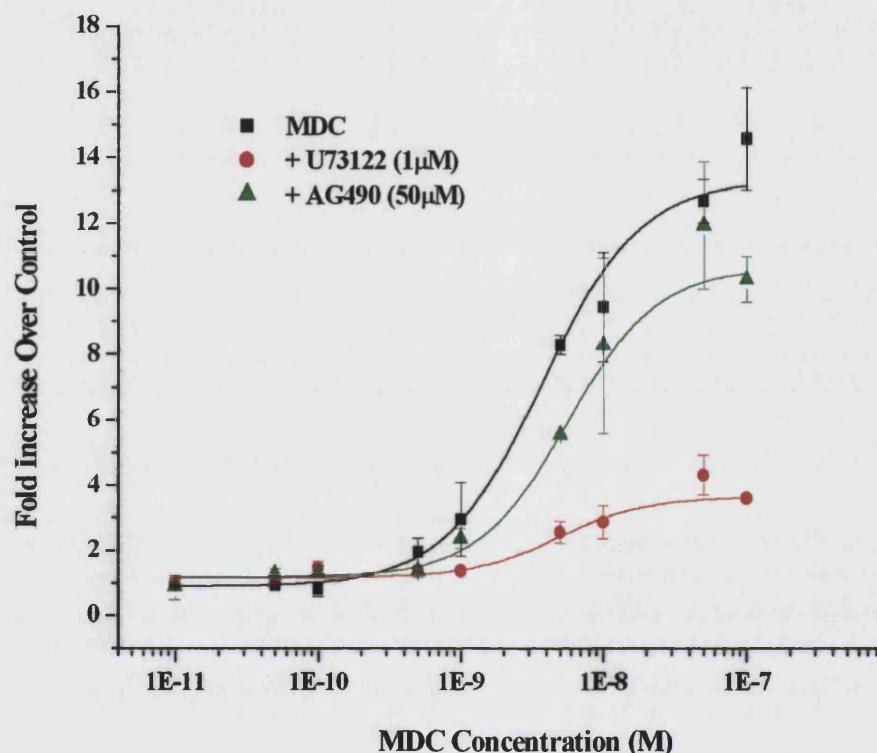


Figure 4.23. Intracellular calcium mobilisation, mediated by MDC, requires PLC activation in Th2 cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with **U73122** (1 μM) or **AG490** (50 μM) for 1 hr. Cells were then labelled with Fluo-4 and aliquoted out at 90,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to MDC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

Figure 4.24. The TARC-induced intracellular calcium flux displays similar sensitivities to inhibitors as that observed for MDC in Th2 cells.

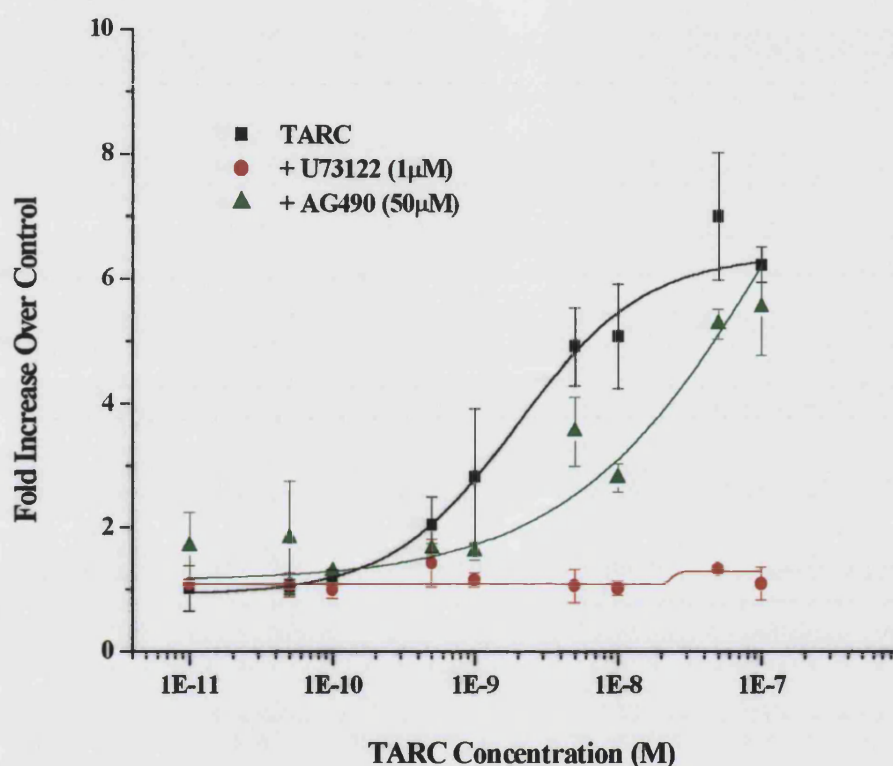


Figure 4.24. The TARC-induced intracellular calcium flux displays similar sensitivities to inhibitors as that observed for MDC in Th2 cells. Levels of intracellular-free calcium were determined using a 96-well plate FLIPR system. Prior to experimentation, cells were washed and resuspended in loading buffer and pretreated with U73122 (1 μ M) or AG490 (50 μ M) for 1 hr. Cells were then labelled with Fluo-4 and aliquoted out at 90,000 cells/well onto poly-L-lysine coated plates, as described in *Materials and Methods*. The cells were then excited at 488 nm using the FLIPR laser and change in fluorescence emission of Fluo-4 determined, in response to TARC stimulation, using a CCD camera with a band-pass interference filter (510–560 nm). Results are expressed as fold increase over unstimulated (vehicle/inhibitor) cells (\pm SEM), with curve fitting performed using the logistic non-linear curve fit equation in Origin. Results are representative of 3 separate experiments.

4.2 Signal Transduction and Directed Cell Migration

MDC- and TARC-Stimulated ERK1/2 Phosphorylation

The ERK-MAPK pathway is a relatively well-characterised and studied cascade that is activated downstream of a plethora of receptor types. The chemokine family of receptors are no different to other groups, with ERK phosphorylation/activation demonstrated in response to a number of chemokines (Kim et al., 2003; Kremer et al., 2003; Lentzsch et al., 2003; Uchida et al., 2003), but not for MDC or TARC. The ERK1/2-MAPK pathway has been implicated in the regulation of cell growth and differentiation, with its activation commonly construed from its phosphorylation (Lewis et al., 1998). The PI3K-PKB and ERK-MAPK modules are two of the pre-eminently studied pathways in a number of experimental systems, including within the chemokine field. The following results have examined the effect of various pharmacological inhibitors, utilised in chemotaxis assays, on these two cascades. These results, along with published data, will allow for a more detailed analysis and construction of the signalling pathways that are potentially downstream of CCR4 and their requirements in directed T cell migration.

MDC and TARC were both able to stimulate the time- and concentration- dependent phosphorylation of ERK1/2 in CHOK1hCCR4 cells, with maximum responses observed between 2 and 5 minutes with optimal chemokine concentrations above 50 nM (fig. 4.25, A). ERK1/2 phosphorylation was additionally witnessed in the leukaemic T cell line CEM and T-lymphoblasts in response to CCR4 ligation (fig. 4.25, B and C). However, the kinetics were slightly more rapid in the T lymphocyte cell types, with peak phosphorylation occurring around 2 minutes. In all three cell types, MDC was consistently more effective than TARC in mediating ERK1/2 phosphorylation. The phorbol ester 12-myristate 13-acetate (PMA) was used as a positive control for ERK phosphorylation.

CCR4-Mediated CEM Cell Chemotaxis is PTX-Dependent

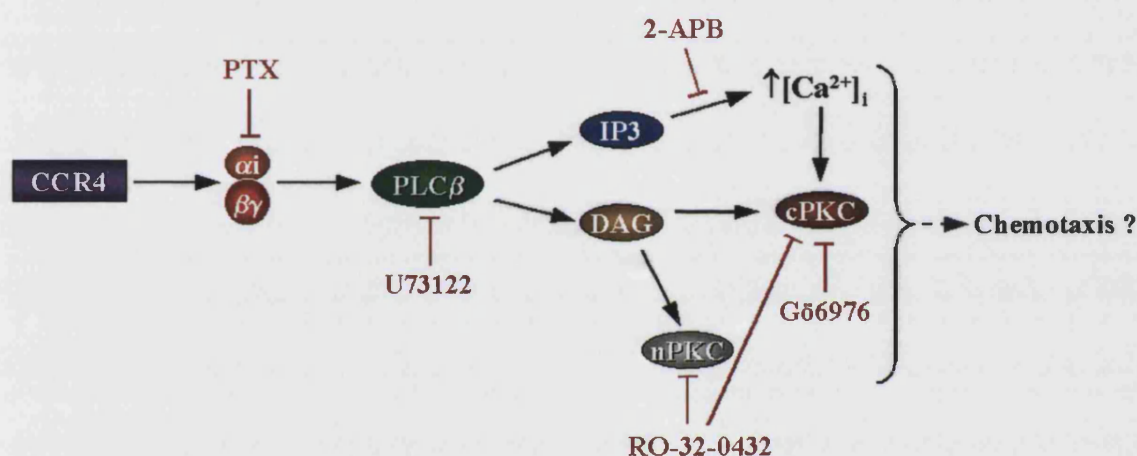
Chemokines were named on a functional basis, due to their ability to direct migration of specific target cell types up their concentration gradient. Hence, the cardinal effect resulting from CCR4 ligation and signalling will be chemotaxis, regardless of cell type. Yet the pathways that are involved in this process are poorly characterised and it is becoming increasingly evident that these required cascades vary between cell- and receptor-type. However, to date all chemokine-mediated directed cell migration has been shown to be sensitive to PTX and pretreatment of CEM cells with 100 ng/ml PTX abrogated MDC-mediated chemotaxis (fig. 4.26, A). The phosphorylation/activation of ERK1/2 and PKB in response to MDC is also dependent upon G α i coupling to CCR4 (fig. 4.26, B and C). A noteworthy observation is the lack of effect of PTX on basal levels of PKB phosphorylation in CEM cells, demonstrating that the treatment was not having an adverse effect on levels of PKB activation in general (fig. 4.26, C).

The role of PI3K in ERK1/2 phosphorylation is controversial. A number of groups have shown that chemokine-stimulated ERK1/2 activation in a variety of leukocytes is sensitive to PI3K inhibition (Knall et al., 1996; Knall et al., 1997; Smit et al., 2003; Sotsios et al., 1999), whilst others disagree and demonstrate that phosphorylation of ERK1/2 is refractory to PI3K inhibition (Curnock et al., 2003; Shyamala and Khoja, 1998; Wain et al., 2002). Pretreatment of CHOK1hCCR4, CEM and T-lymphoblast cells with 10 μ M LY294002 has no effect on MDC-mediated ERK1/2 phosphorylation (fig. 4.27). The effect of PI3K inhibition on PKB phosphorylation and CCR4-induced T cell migration has been discussed earlier (see 'Results I').

Role of PLC, Calcium and PKC on MDC-Mediated Directed CEM Cell Migration

The activation of PLC sensitive signalling pathways have been demonstrated for a number of chemokines, including CXCL8/CXCR2, CCL15/CCR1 and CCL2/CCR2 (Bacon et al., 1995; Kim et al., 2003; Kuang et al., 1996; Li et al., 2000). Additionally, two recent studies has demonstrated the importance of PLC, using the inhibitor U73122, for

CXCL12/CXCR4- and CXCL11/CXCR3-mediated chemotaxis of T cells (Smit et al., 2003; Soriano et al., 2003). U73122 pretreatment of CEM cells concentration-dependently inhibited MDC-induced chemotaxis with abrogation occurring with 3 μ M U73122 (fig. 4.28, A). Furthermore, U73122 inhibited PKB phosphorylation in response to MDC with U73433, the inactive isomer, having no effect (fig. 4.28, B). Smit, et al. (Smit et al., 2003) demonstrate U73122 having a moderate reduction on CXCL11 stimulated ERK1/2 phosphorylation in T-lymphocytes and with complete inhibition observed in CXCR3 transfected COS-7 cells. In CEM cells, MDC-stimulated ERK1/2 phosphorylation was slightly curtailed at higher concentrations of U73122 but there was also an accompanying increase in basal levels of phosphorylation (fig. 4.28, B). The net effect was a similar level of phosphorylation observed for both unstimulated and stimulated cells at 10 μ M U73122.



So far the chemotaxis data has displayed a similar sensitivity towards the inhibitors as was observed for CCR4-induced calcium flux responses. Studies to date have suggested that in the absence of adhesive molecules calcium is not required for chemotaxis (Alteraifi and Zhelev, 1997; Elferink et al., 1992; Fabbri et al., 1997; Kuijpers et al., 1992). Conversely, chemotaxis of neutrophil in the presence of adhesive molecules appears to depend on intracellular calcium (Alteraifi and Zhelev, 1997; Hofman et al., 1999; Mandeville and Maxfield, 1997). Analysis of CEM cell chemotaxis with U73122 has hinted that calcium and/or DAG are likely to be required for CCR4-mediated migration. Pretreating CEM cells with 2-APB, at concentrations that abrogate CCR4-mediated intracellular calcium mobilisation (fig. 4.15), had no impact upon MDC-induced chemotaxis (fig. 4.29, A). This correlates with the theory that calcium is not required for directed cell migration in the

absence of adhesive molecules. Moreover, 2-APB treatment had no influence on PKB phosphorylation in response to MDC but did severely reduce ERK1/2 phosphorylation events in a concentration-dependent manner (fig. 4.29, *B*).

So far, use of inhibitors has indicated that calcium is a dispensable signal for cell migration, whilst the PLC inhibitor has a significant effect on migration. One explanation for this data could be that the DAG/PKC pathway plays an important role. The most widely studied DAG effector is PKC. The PKC is a very well studied kinase in many different experimental settings, including chemotaxis. It has been demonstrated that various PKC isoforms are required for chemotaxis of various cell types in response to a variety of stimuli (Carnevale and Cathcart, 2003; Entschladen et al., 2000; Etienne-Manneville and Hall, 2001; Laudanna et al., 1998). Gö6976 is a selective inhibitor for the classical protein kinase Cs (i.e. α , β and γ) and effective at nanomolar concentrations, whilst novel PKC isoform activity (i.e. δ , ϵ , η and θ) is unaffected, even at micromolar concentrations (Martiny-Baron et al., 1993). Reassuringly, pretreatment of CEM cells with Gö6976, at concentrations up to 300 nM, had no influence on MDC-induced directed cell migration (fig. 4.30, *A*). Indeed, Gö6976 had no detrimental effect on ERK1/2 and PKB phosphorylation in response to MDC stimulation either (fig. 4.30, *B*). This data compares favourably with the lack of requirement for calcium mobilisation for MDC-induced CEM cell chemotaxis, as the classical PKC isoforms require both calcium and DAG for activation. In contrast, the novel PKC isoforms require only DAG for their activation. Unfortunately, there are no pharmacological tools to specifically constrain the activity of these isoforms individually. However, there are a number of inhibitors that display inhibitory effects over both the classical and novel isoforms. One of these, RO-32-0432, has been described as a broad-spectrum PKC isoform inhibitor, although it does display a 10-fold and 4-fold more selective action for PKC α (IC_{50} = 9 nM) and PKC β (IC_{50} = 28 nM) over PKC ϵ (IC_{50} = 108 nM), respectively (Wilkinson et al., 1993). Pretreatment of CEM cells with 10 μ M RO-32-0432 (30 min) completely abated MDC-driven CEM cell chemotaxis (fig. 4.31, *A*), whilst ERK1/2 phosphorylation was refractory to the inhibitory effects of this compound (fig. 4.31, *B*). However, PKB phosphorylation in response to MDC was partially down-regulated after RO-32-0432 treatment (fig. 4.31, *B*). There is a single pharmacological inhibitor available to study the effect of an individual novel PKC

isoform, namely rottlerin and PKC δ . This inhibitor displays up to 10-fold greater selectivity for PKC δ ($IC_{50} = 3 - 6 \mu M$) over other isoforms ($IC_{50} = 30 - 42 \mu M$) (Gschwendt et al., 1994). PKC δ has recently been implicated having a role in the chemotaxis of human osteogenic sarcoma (HOS) cells to CCL15 (CCR1) (Ko et al., 2002; Li et al., 2003a). Rottlerin inhibited MDC-mediated CEM cell chemotaxis in a concentration-dependent manner, with complete inhibition observed at 10 μM rottlerin (fig. 4.32, A). ERK1/2 phosphorylation was refractory to PKC δ inhibition, and in contrast to RO-32-0432, so was PKB phosphorylation (fig. 4.32, B). PKC δ has been shown to be a ubiquitously expressed enzyme and immunoblotting cell lysates for PKC δ confirmed the expression of this isozymes in CEM cells (fig. 4.32, C) (Liu and Heckman, 1998).

Figure 4.25. MDC and TARC stimulate the phosphorylation of the ERK-MAPK in a concentration- and time-dependent manner.

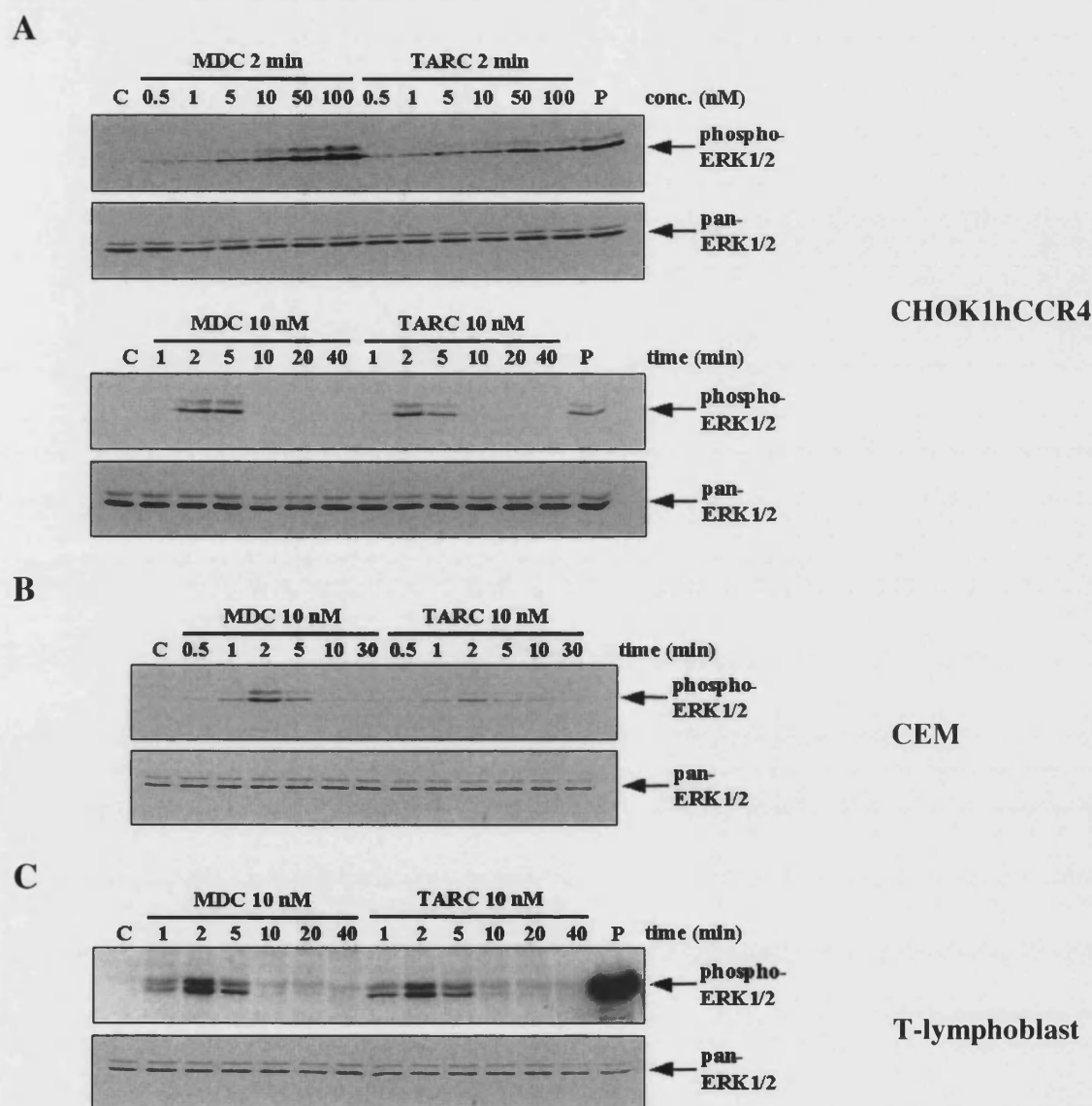


Figure 4.25. MDC and TARC stimulate the phosphorylation of the ERK-MAPK in a concentration- and time-dependent manner. Levels of phospho-ERK1/2 protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in appropriate media, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. CHOK1hCCR4 (A), CEM (B) or T-lymphoblast (C) cells were then stimulated at 37°C with indicated concentrations of ligand for 2 mins or for the indicated times, and cells lysed by the addition of 1 x sample buffer. PMA (100nM and 5 min), P, was used as a positive control for ERK1/2 phosphorylation. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK1/2 Ab with affinity for the Thr 202- and Tyr 204-phosphorylated, active form, of ERK1/2, and protein was visualised with ECL. Stripping and reprobing blots with anti-ERK1/2 Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 3 separate experiments.

Figure 4.26. CCR4-mediated signalling events and chemotaxis are pertussis toxin-sensitive.

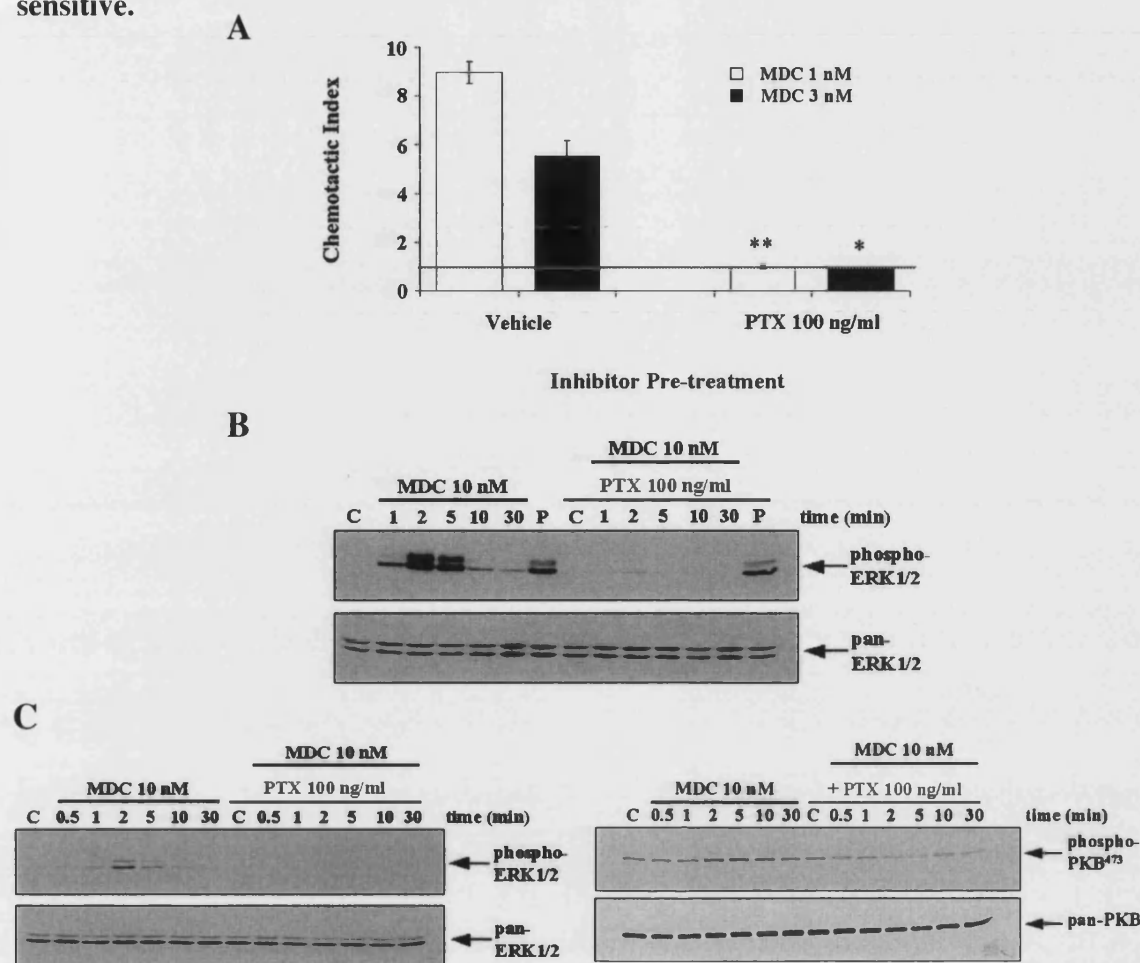


Figure 4.26. CCR4-mediated signalling events and chemotaxis are pertussis toxin-sensitive. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 or 3 nM MDC. Cells were preincubated with the 100 ng/ml pertussis toxin (PTX) for 16 hours, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of PTX (*, $p < 0.0001$; **, $p < 0.00005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CHOK1hCCR4 (B) and CEM (C) cells treated and untreated with pertussis toxin were stimulated with MDC (10 nM) for indicated times (C = vehicle 5 min; P = 100 nM PMA 5 min), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.27. MDC-induced phosphorylation of ERK1/2 does not require PI3K activity.

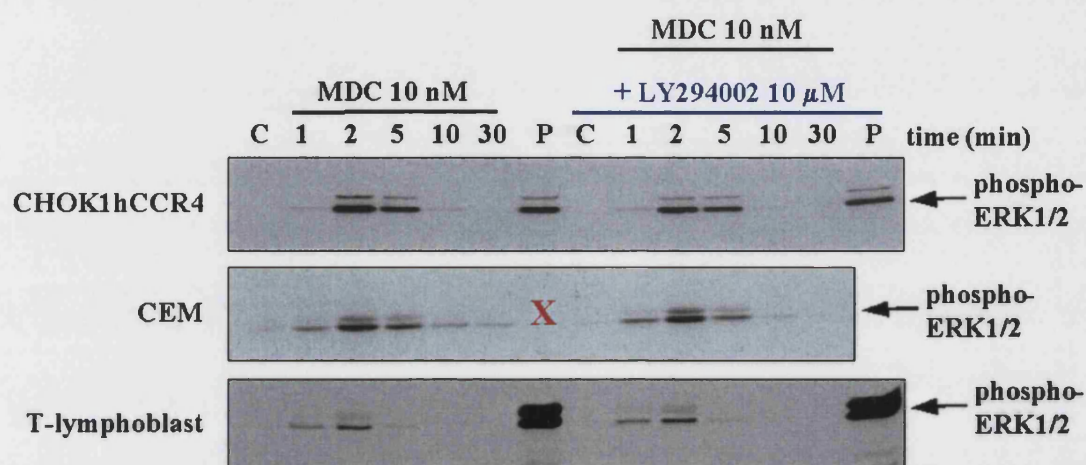


Figure 4.27. MDC-induced phosphorylation of ERK1/2 does not require PI3K activity. Levels of phospho-ERK1/2 protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in appropriate media, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC for indicated times (in the presence or absence of $10 \mu\text{M}$ LY294002, 30 min preincubation), and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific ERK1/2 Ab with affinity for the Thr 202- and Tyr 204-phosphorylated, active form, of ERK1/2, and protein was visualised with ECL. Stripping and reprobing blots with anti-PKB Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 3 separate experiments. Key: P, 100 nM PMA 5 min. The X indicates there was no sample in that lane.

Figure 4.28. CCR4-mediated CEM cell chemotaxis and ERK1/2 phosphorylation require PLC activation.

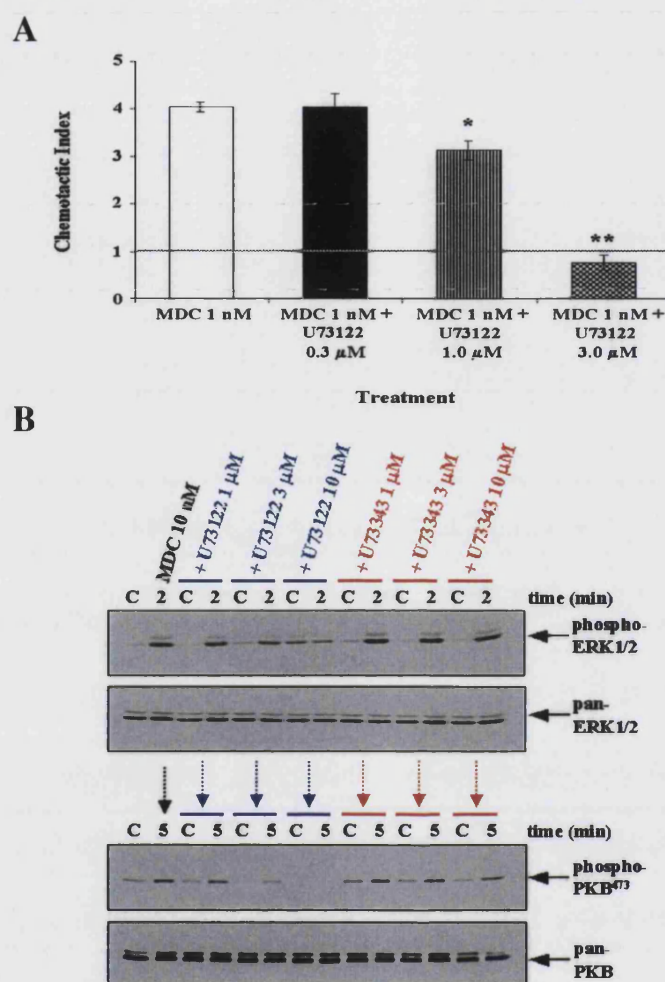


Figure 4.28. CCR4-mediated CEM cell chemotaxis and ERK1/2 phosphorylation requires PLC activation. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of U73122 for 1 hour, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of U73122 (*, $p < 0.05$; **, $p < 0.000005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B) cells treated and untreated with U73122, or the negative control U73343, were stimulated with MDC (10 nM) for indicated times (C = vehicle), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.29. MDC-induced CEM cell chemotaxis does not require calcium mobilisation.

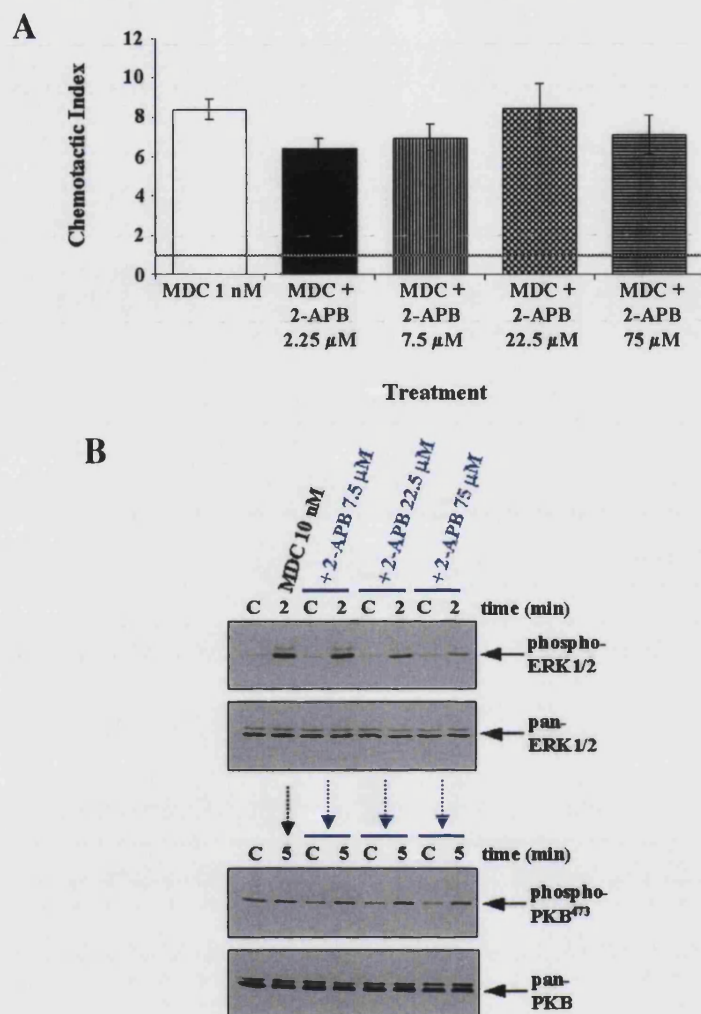


Figure 4.29. MDC-induced CEM cell chemotaxis does not require calcium mobilisation. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of 2-APB for 1 hour, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test ($p < 0.05$) to compare responses in the presence and absence of 2-APB. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B) cells treated and untreated with 2-APB were stimulated with MDC (10 nM) for indicated times (C = vehicle), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.30. Classical PKC isoform activity is not required for CCR4-mediated CEM cell chemotaxis.

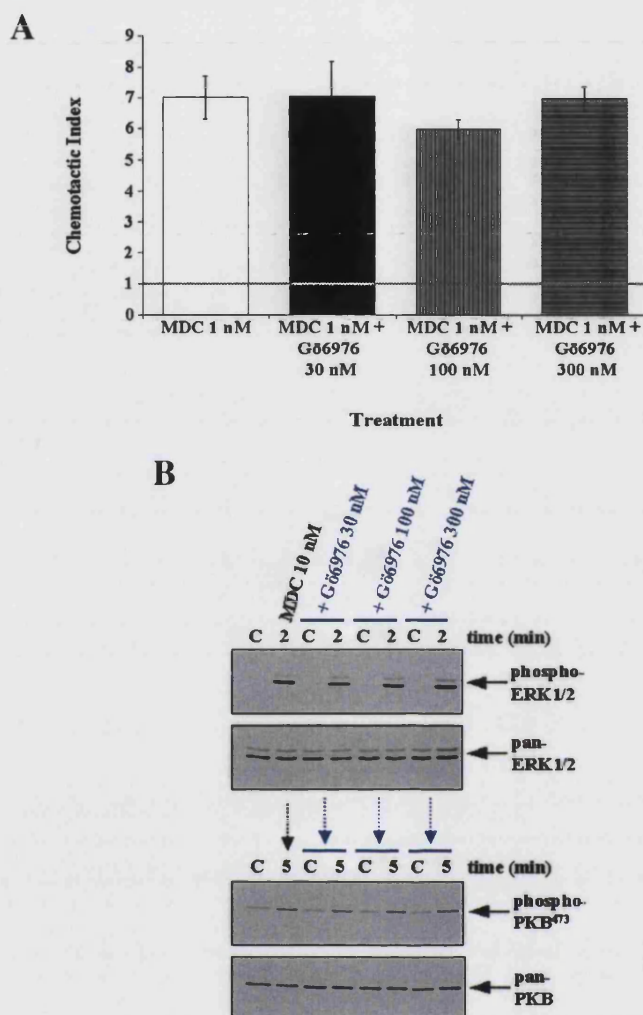


Figure 4.30. Classical PKC isoform activity is not required for CCR4-mediated CEM cell chemotaxis. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of Gö6976 for 30 min, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test ($p < 0.05$) to compare responses in the presence and absence of Gö6976. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B) cells treated and untreated with Gö6976 were stimulated with MDC (10 nM) for indicated times (C = vehicle), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.31. MDC-induced CEM cell chemotaxis is sensitive to a broad spectrum PKC isoform inhibitor.

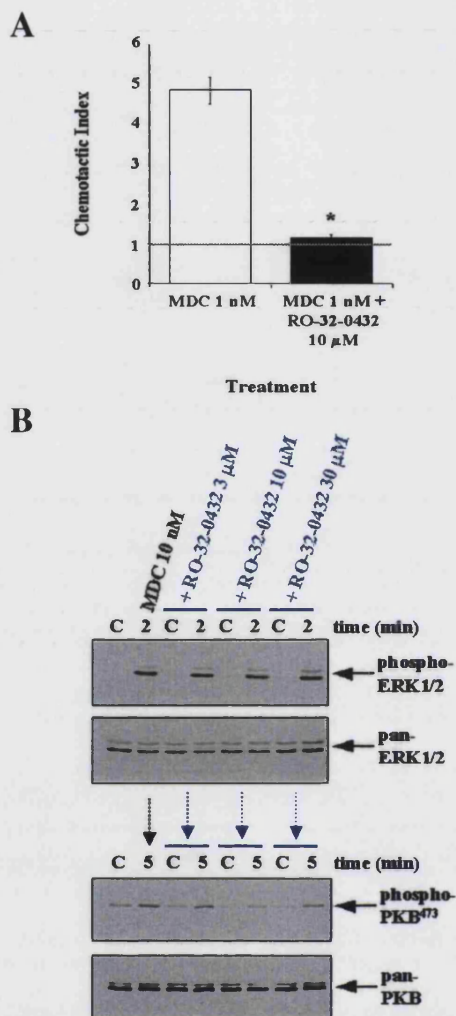


Figure 4.31. MDC-induced CEM cell chemotaxis is sensitive to a broad spectrum PKC isoform inhibitor. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of RO-32-0432 for 30 min, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction (*, $p < 0.00005$) to compare responses in the presence and absence of RO-32-0432. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B) cells treated and untreated with RO-32-0432 were stimulated with MDC (10 nM) for indicated times (C = vehicle), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.32. Rottlerin pre-treatment inhibits MDC-induced CEM cell chemotaxis.

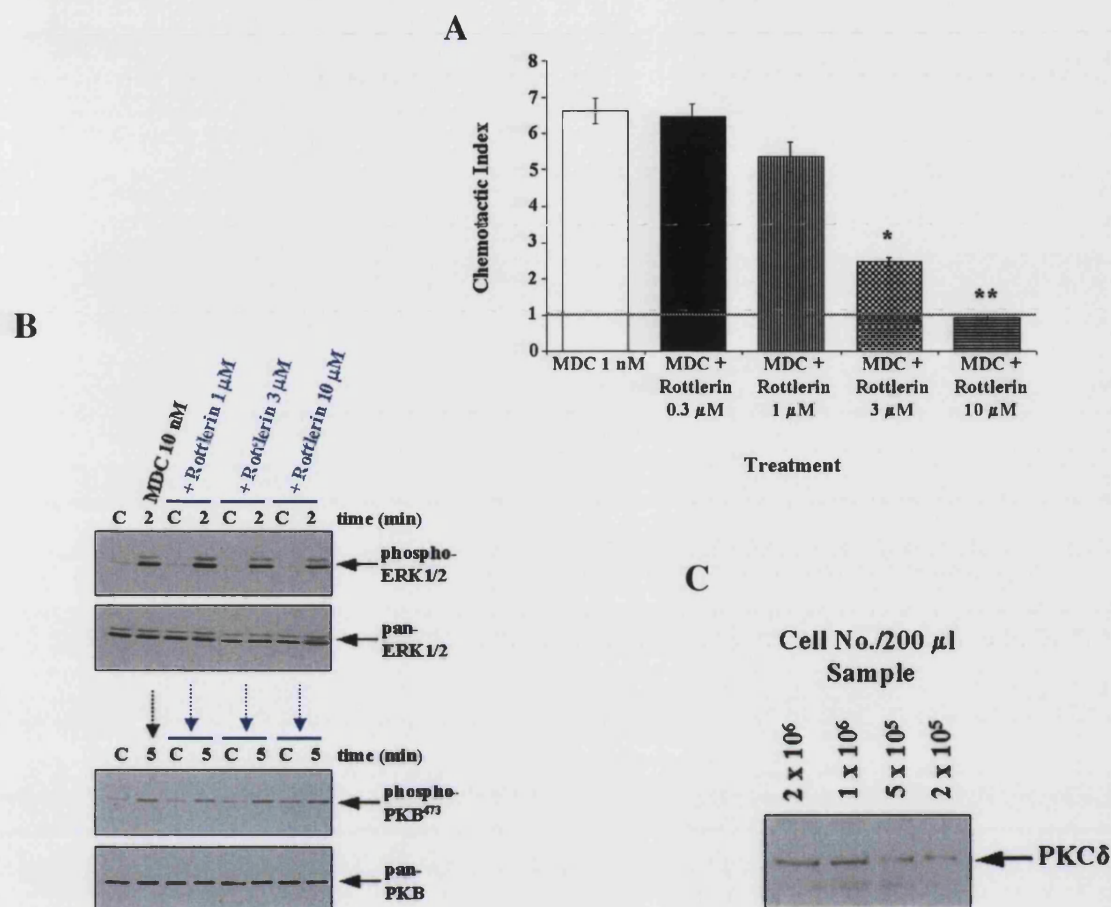
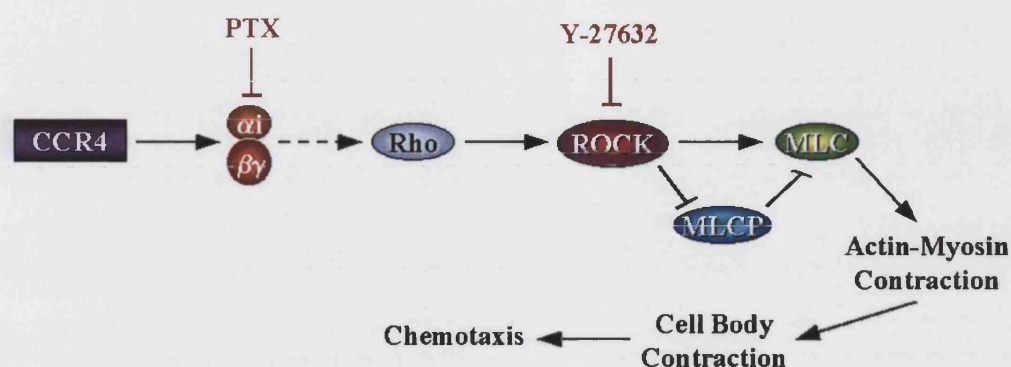


Figure 4.32. Rottlerin pre-treatment inhibits MDC-induced CEM cell chemotaxis. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. **A**, Cells were preincubated with indicated concentrations of rottlerin for 30 min, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction (*, $p < 0.00005$; **, $p < 0.000005$) to compare responses in the presence and absence of rottlerin. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. **B**, CEM cells treated and untreated with rottlerin were stimulated with MDC (10 nM) for indicated times (C = vehicle), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. **C**, CEM cell lysates with varying amounts of protein (i.e. cell numbers) were additionally immunoblotted for expression of PKC δ , with a PKC δ Ab. Results are representative of 3 separate experiments.

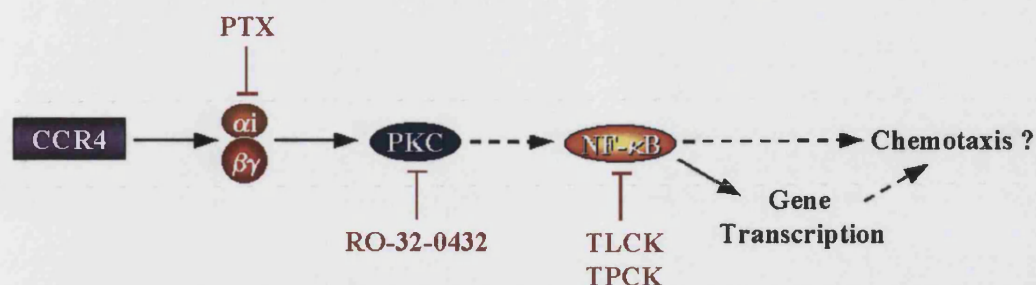
ROCK Performs a Pivotal Function in MDC-Induced CEM Cell Chemotaxis



Recent attention has focused on the role of Rho GTPase and its downstream effector Rho-dependent kinase (ROCK) in chemokine-elicited migration, as it appears to be required for CCR7 and CXCR4 T lymphocyte migration in response to CCL19/CCL21 and CXCL12 respectively (Bardi et al., 2003; Vicente-Manzanares et al., 2002). Pretreatment of CEM cells with the ROCK inhibitor Y-27632 markedly inhibited MDC-induced chemotaxis in a concentration-dependent manner (fig. 4.33, A). Y-27632 had no effect on the ability of MDC to elicit the phosphorylation of ERK1/2 or PKB (fig. 4.33, B).

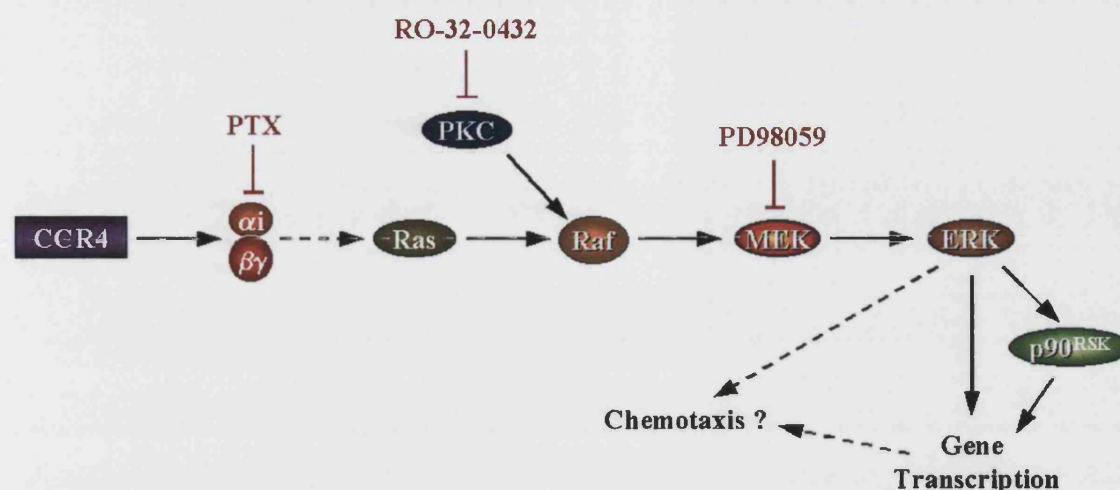
ROCK is known to inhibit myosin light chain phosphatase and to directly phosphorylate myosin light chain (Raftopoulou and Hall, 2004). Hence, we verified that Y-27632 did indeed inhibit the intended ROCK target by monitoring cellular levels of phospho-MLC2^{Ser19} in lysates derived from resting and CCL22-stimulated CEM cells. CCL22 induced a sustained phosphorylation of MLC over a 30 minute period, which was completely attenuated by pre-treatment with Y-27632 (fig. 4.34).

The Two Protease Inhibitors TLCK and TPCK Abrogate Directed T Cell Migration



These two chloromethyl ketones have a broad-spectrum of activity against various serine proteases. TLCK and TPCK (effective concentrations of both are 10 – 100 μ M) have been demonstrated to inhibit NF- κ B activation, with subsequent transcription of the iNOS gene, and p70^{S6K} activation (Breithaupt et al., 1996; Grammer and Blenis, 1996; Kim et al., 1995; Schini-Kerth et al., 1997). NF- κ B has been demonstrated to be activated by chemokine receptor ligation and, with the use of TLCK/TPCK and SN50 (a peptide inhibitor of NF- κ B), been shown to be required for optimal migratory responses (Altura and Gebrewold, 2002; Barna and Kew, 1995; Cherla and Ganju, 2001; Ko et al., 2002; Zerneck et al., 2001). Studies in various systems have suggested a plethora of mechanisms for the activation of the NF- κ B pathway, including via PKC δ (Page et al., 2003). Treatment of CEM cells with TLCK or TPCK, at various concentrations for 1 hour prior to chemokine exposure in a modified Boyden chamber, completely inhibited directed T cell migration in a concentration-dependent manner (fig. 4.35, A and B). In addition, both compounds inhibited PKB phosphorylation in response to MDC, and TLCK had a slight detrimental effect on ERK1/2 phosphorylation (fig. 4.36). In contrast, TPCK pretreatment of CEM cells had no ramifications on chemokine stimulated ERK1/2 phosphorylation but did ameliorate basal levels to those of MDC-stimulated intensities at the higher concentrations of inhibitor (fig. 4.36). The NOS inhibitor L-NAME has been utilised in a study that reported the requirement of NO in CXCL12/CXCR4-mediated Jurkat T cell chemotaxis (Cherla and Ganju, 2001). However, L-NAME pretreatment of CEM cells had no influence on either MDC-mediated chemotaxis or ERK1/2 phosphorylation, even at concentrations as high as 2 mM (fig. 4.37, A and B).

MEK Inhibition is Inconsequential for CEM Cell Chemotaxis



So far several inhibitors have demonstrated inhibitory outcomes on both ERK1/2 and CEM cell chemotaxis in response to MDC. However, this gives little indication to the actual requirement of ERK1/2 for directed cell migration. MEK1/2 is the upstream activator of ERK1/2 and its only known substrate. A widely used inhibitor of MEK to study ERK1/2-mediated responses is PD98059. PD98059 does not compete for the ATP-binding site of MEK or its association with ERK1/2 but is instead thought to inhibit through an allosteric mechanism (Alessi et al., 1995; Dudley et al., 1995). There are conflicting reports for the involvement of MEK/ERK in chemokine-mediated cell migration. Published studies have attenuated CXCL12-, CCL20-, CCL2-, CCL7-, CCL8-, CCL11- and CCL11-induced actin polymerisation and/or cell migration of leukocytes (Sotsios et al., 1999; Sullivan et al., 1999b; Wain et al., 2002). Whilst other studies have demonstrated that CXCL12-, CXCL1-, CCL2- and CXCL11-induced leukocyte chemotaxis does not depend on ERK1/2 activation (Ashida et al., 2001a; Cherla and Ganju, 2001; Smit et al., 2003; Wang et al., 2002a). PD98059 treated CEM cells chemotaxed normally to MDC (fig. 4.38, A), whilst the same concentration of PD98059, as expected, abolished ERK1/2 phosphorylation in response to MDC in CEM (fig. 4.38, B), CHOK1hCCR4 (fig. 4.38, C) and T-lymphoblast (fig. 4.38, D) cells. There was no unfavourable impact on PKB phosphorylation in any of the cell types tested (fig. 4.38, C and D).

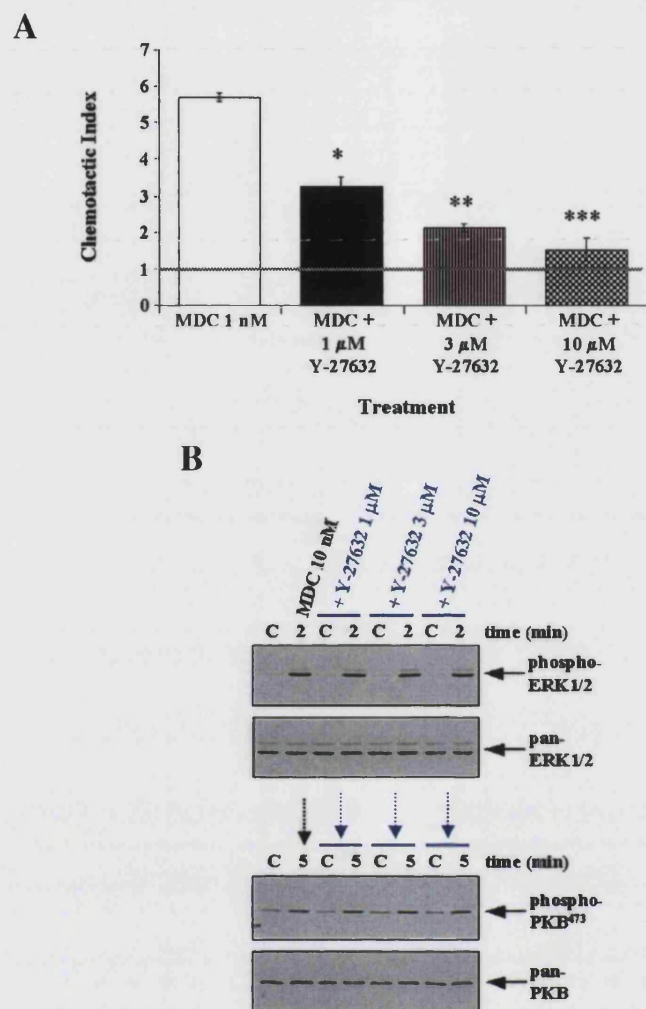
Figure 4.33. MDC-induced CEM cell chemotaxis is sensitive to a ROCK inhibitor.

Figure 4.33. MDC-induced CEM cell chemotaxis is sensitive to a ROCK inhibitor. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of Y-27632 for 1 hour, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction (*, $p < 0.0005$; **, $p < 0.00005$; ***, $p < 0.000005$) to compare responses in the presence and absence of Y-27632. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B) cells treated and untreated with Y-27632 were stimulated with MDC (10 nM) for indicated times (C = vehicle), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.34. MDC phosphorylates MLC2 in CEM cells in a ROCK-dependent manner.

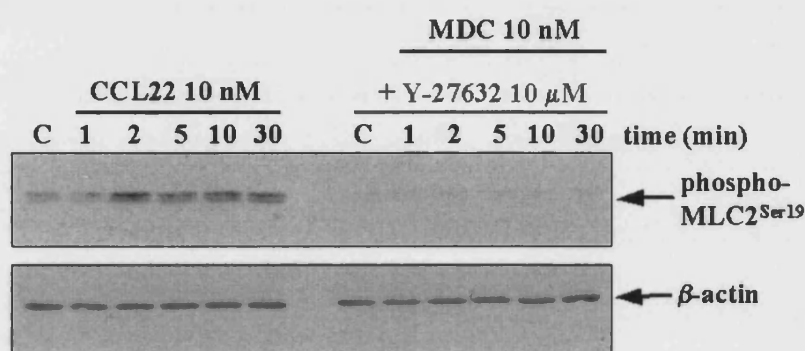
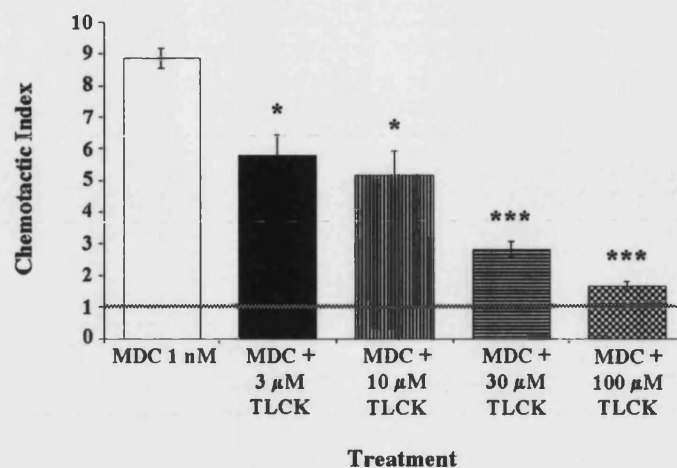


Figure 4.34. MDC phosphorylates MLC2 in CEM cells in a ROCK-dependent manner. Levels of phospho-MLC2 protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in appropriate media, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC for indicated times (in the presence or absence of 10 μ M Y-27632, 60 min preincubation), and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with a phospho-specific MLC2 Ab with affinity for the Ser 19-phosphorylated form of MLC2, and protein was visualised with ECL. Stripping and reprobing blots with anti- β -actin Ab confirmed equal loading, as described in *Materials and Methods*. Data representative of 3 separate experiments.

Figure 4.35. Both TLCK and TPCK are able to attenuate CCR4-mediated CEM cell chemotaxis.

A



B

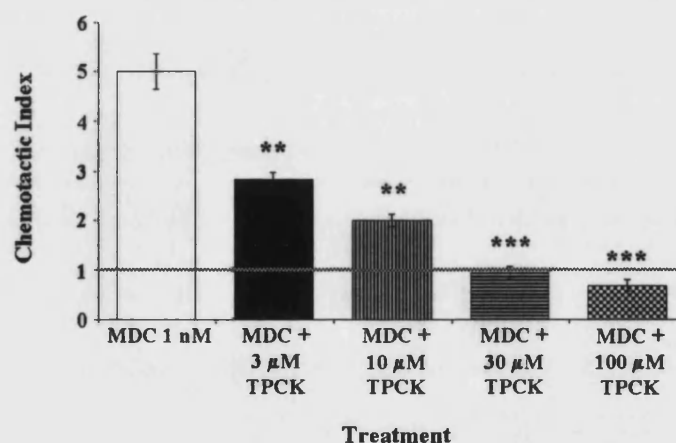


Figure 4.35. Both TLCK and TPCK are able to attenuate CCR4-mediated CEM cell chemotaxis. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of TLCK, A, or TPCK, B, for 1 hour, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.00005$) to compare responses in the presence and absence of TLCK or TPCK. The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Figure 4.36. MDC-induced PKB but not ERK1/2 phosphorylation is sensitive to TLCK and TPCK pre-treatment.

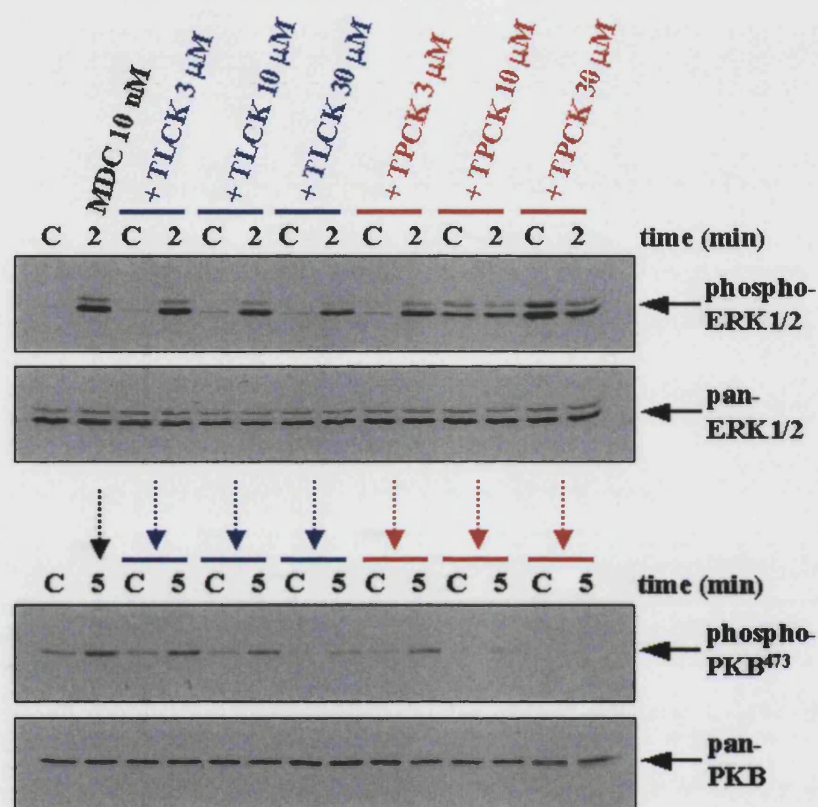


Figure 4.36. MDC-induced PKB but not ERK1/2 phosphorylation is sensitive to TLCK and TPCK pre-treatment. Levels of phospho-PKB or -ERK1/2 protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in appropriate media, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC for indicated times (in the presence or absence of indicated concentrations of TLCK or TPCK, 60 min preincubation), and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots were stripped and reprobed with the appropriate pan-Ab to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Figure 4.37. NOS is not involved in MDC-mediated CEM cell chemotaxis.

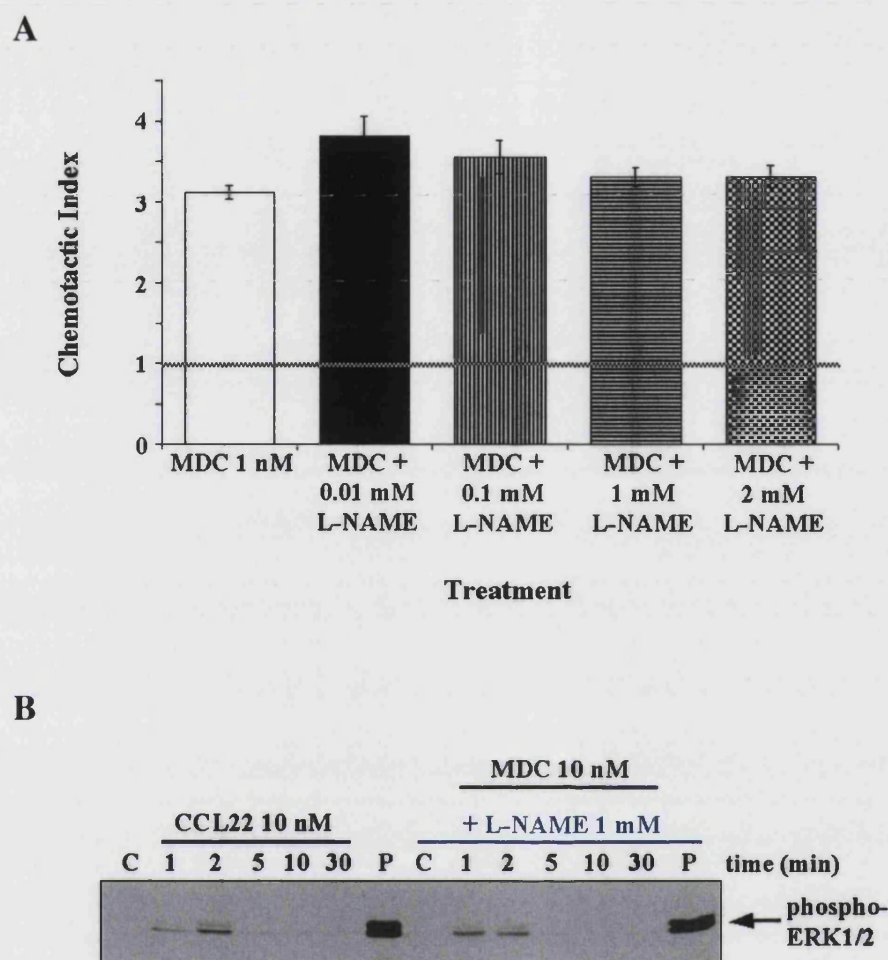


Figure 4.37. NOS is not involved in MDC-mediated CEM cell chemotaxis. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of L-NAME for 1 hour, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test to compare responses in the presence and absence of L-NAME ($p < 0.05$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B) cells treated and untreated with L-NAME were stimulated with MDC (10 nM) for indicated times (C = vehicle; P = 100 nM PMA 5 min), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab. Results are representative of 3 separate experiments.

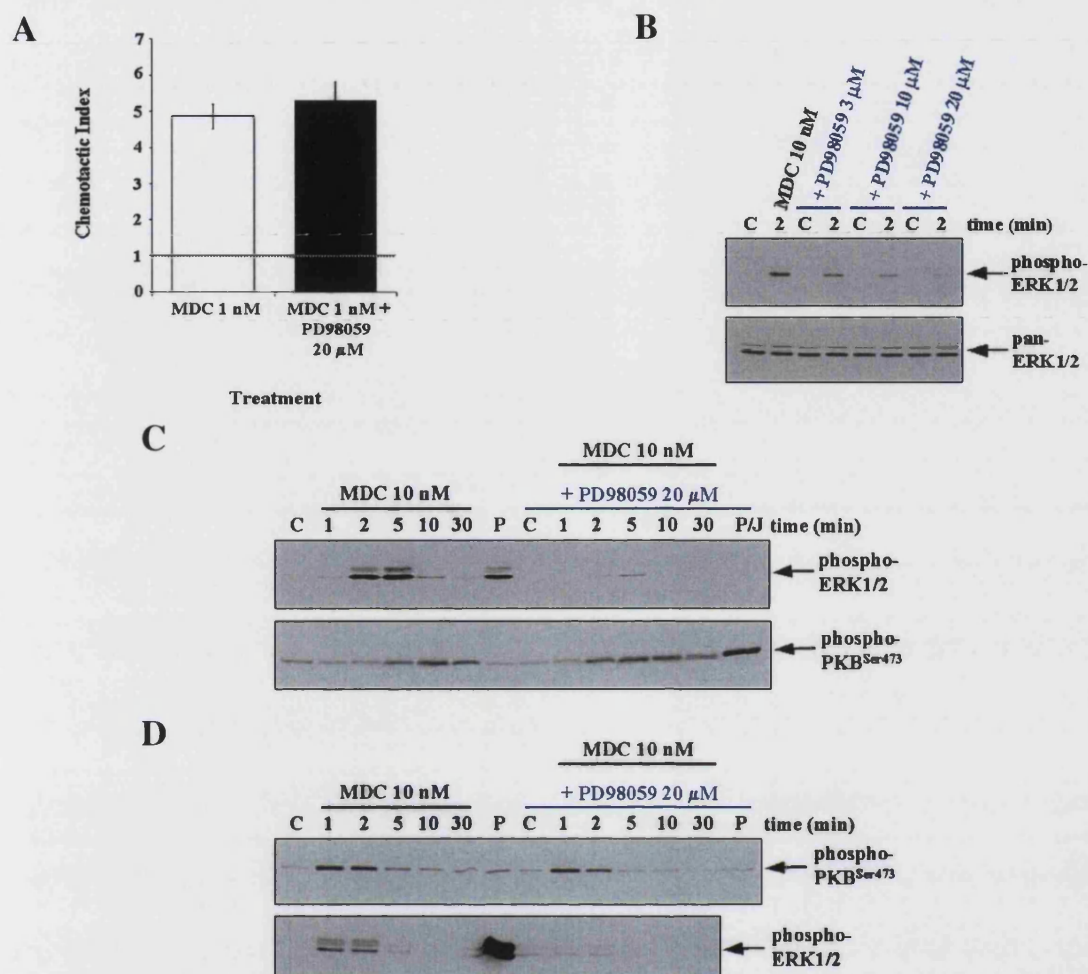
Figure 4.38. MEK inhibition has no effect upon MDC-induced CEM cell chemotaxis.

Figure 4.38. MEK inhibition has no effect upon MDC-induced CEM cell chemotaxis. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of PD98059 for 1 hour, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test to compare responses in the presence and absence of PD98059 ($p < 0.05$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. CEM (B), CHOK1hCCR4 (C) or T-lymphoblast (D) cells treated and untreated with PD98059 were stimulated with MDC (10 nM) for indicated times (C = vehicle; P = 100 nM PMA 5 min; J = Jurkat cell lysate sample), and lysates generated. These lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB^{Ser473} Ab. The blots (B) were stripped and reprobed with pan-ERK1/2 to verify equal loading and efficiency of protein transfer. Results are representative of 3 separate experiments.

Effect of Various Other Kinase Inhibitors on CCR4-Induced Chemotaxis

A couple of other molecules that have been demonstrated in published papers to be required for directed cell migration were also studied in chemotaxis assays, namely JAK2/3 and p38. JAK2/3 has been shown to be essential for CXCL12-mediated T cell chemotaxis by inhibition with 50 μ M AG490 (Soriano et al., 2003), yet 100 μ M AG490 had no effect on CEM cell chemotaxis in response to MDC (fig. 4.39, A). Likewise, p38 (a MAPK) has been implicated in directed cell migration (Adachi et al., 2001; Ashida et al., 2001a; Cara et al., 2001), but pretreating CEM cells with SB203580, at a concentration used in the above-referenced studies and others, had no effect on MDC-mediated T cell chemotaxis (fig. 4.39, A). However, both MDC and TARC are able to phosphorylate p38 in CEM cells in a time-dependent manner upon chemokine stimulation (fig. 4.39, B).

Th2 Cell Chemotaxis Requires Similar Pathway Activation to CEMs

CEMs are a transformed human leukaemic T cell line that, by definition, is not a physiologically normal cell type. As in the first results chapter ('Results I'), human Th2 cells were generated from PBMCs that were differentiated *in vitro* into Th2-like cells and used in chemotaxis assays to confirm that data obtained with CEM cells were likely to be 'normal' events. MDC- and TARC-mediated Th2 cell chemotaxis was wholly sensitive to PTX (fig. 4.40) and U73122 pretreatment (fig. 4.41), but insensitive to 2-APB-mediated inhibition of intracellular calcium mobilisation (fig. 4.41). Incidentally, the negative analogue of U73122, U73343, had no repercussions on chemotaxis after pretreatment of the Th2 cells (fig. 4.41). In addition, 10 μ M RO-32-0432 abolished CCR4-induced Th2 cell chemotaxis (fig. 4.42, A), and partially negated MDC-induced PKB phosphorylation in T-lymphoblasts (fig. 4.42, B). Both RO-32-04 and rottlerin pretreatment had no detrimental influence on ERK1/2 and PKB phosphorylation in response to MDC stimulation in T-lymphoblasts, respectively (fig. 4.42, B). The serine protease inhibitors TLCK and TPCK had massive ramifications on Th2 cell chemotaxis upon preincubation, with an almost complete cessation of any cell movement (fig. 4.43). In contrast, CCR4-mediated Th2 cell chemotaxis was refractory to the inhibitory effects of AG490 (JAK2/3) and WHI-P154 (JAK3) on their target kinases (fig. 4.44). These results correlate very positively with those obtained for CEM cells, with little difference in sensitivity to the utilised inhibitors.

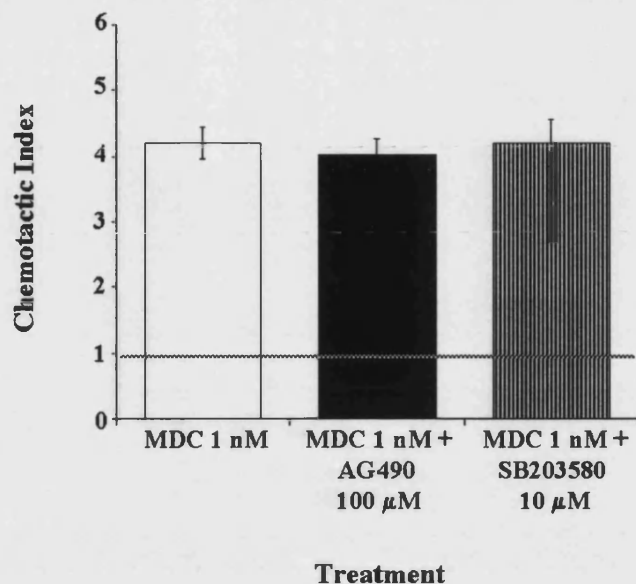
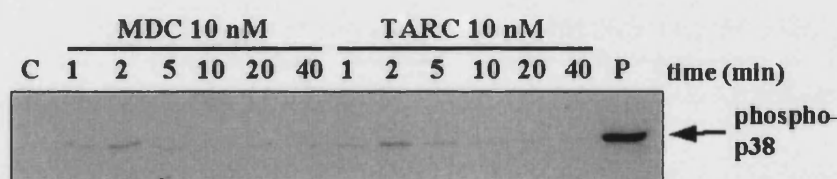
Figure 4.39. Sensitivity of CCR4-mediated CEM cell chemotaxis to various inhibitors.**A****B**

Figure 4.39. Sensitivity of CCR4-mediated CEM cell chemotaxis to various inhibitors. CEM cells (2×10^5 cells/200 μ l) were added to the upper wells of a reusable MB series 96-well chemotaxis chamber, above lower wells containing 1 nM MDC. Cells were preincubated with indicated concentrations of AG490 or SB203580 for 1 hour, A, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.5 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with quintuplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating (+/- inhibitor). Data were analysed by ANOVA and Student's *t* test to compare responses in the presence and absence of inhibitor ($p < 0.05$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Levels of phospho-p38 protein were determined by western blot analysis. CEM cells were washed three times in appropriate media, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC or TARC for indicated times and cells lysed by the addition of 1 \times sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho p38 Ab, with affinity for Thr 180- and Tyr 182-phosphorylated form. Results are representative of 2 separate experiments.

Figure 4.40. MDC- and TARC-mediated Th2 cell chemotaxis is sensitive to pertussis toxin pretreatment.

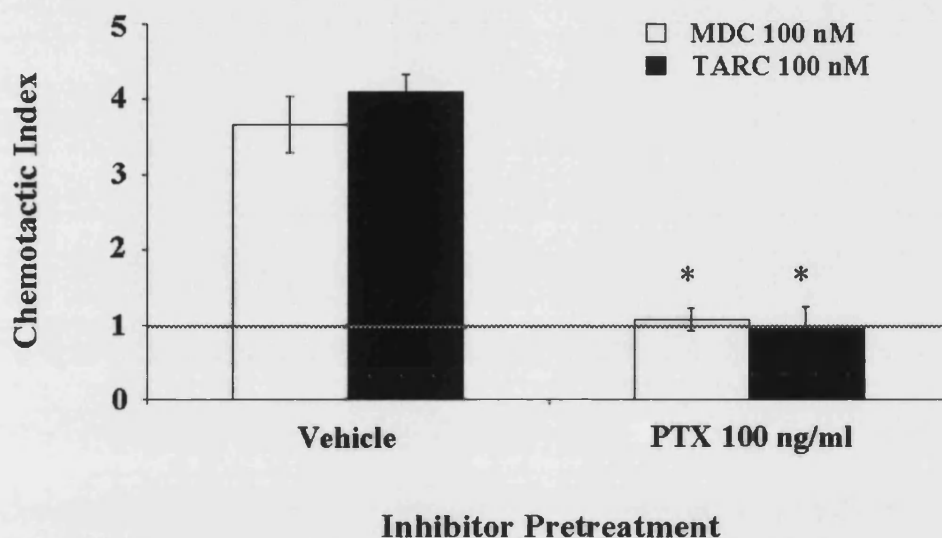


Figure 4.40. MDC- and TARC-mediated Th2 cell chemotaxis is sensitive to pertussis toxin pretreatment. Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells were preincubated with 100 ng/ml pertussis toxin (PTX) for 16 hours. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing 100 nM MDC or 100 nM TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of PTX (*, $p < 0.001$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Figure 4.41. CCR4-mediated Th2 cell chemotaxis is PLC-dependent but calcium independent.

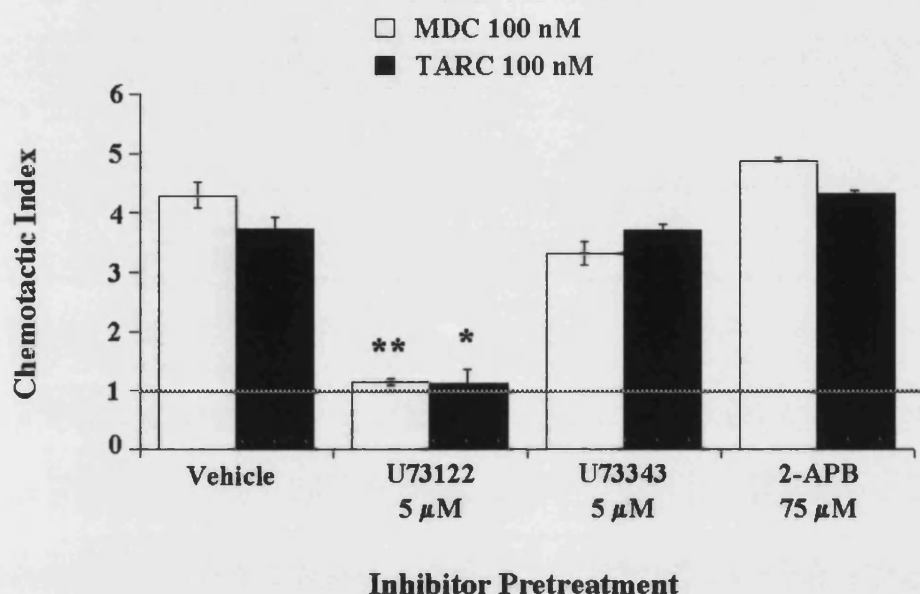


Figure 4.41. CCR4-mediated Th2 cell chemotaxis is PLC-dependent but calcium independent. Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells were preincubated with U73122, U73343 or 2-APB for 1 hour, at indicated concentrations. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing 100 nM MDC or 100 nM TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of inhibitor (*, $p < 0.005$; **, $p < 0.0005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Figure 4.42. CCR4-mediated Th2 cell chemotaxis is sensitive to the inhibition of PKC isoforms.

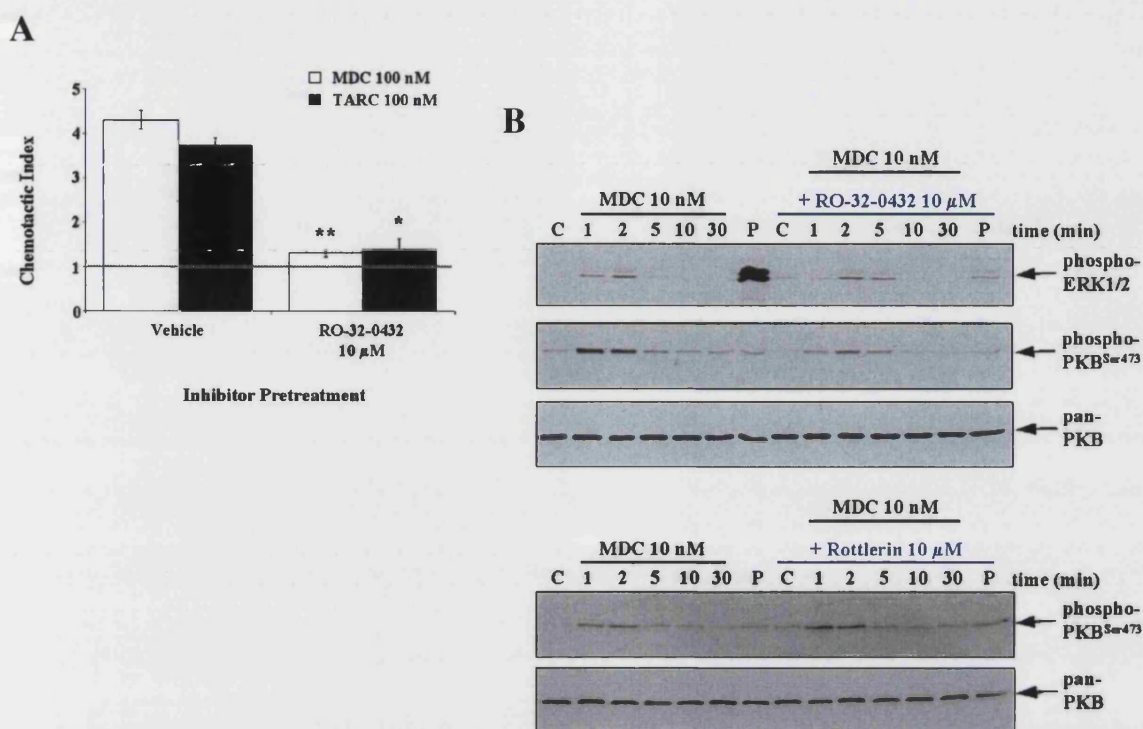


Figure 4.42. CCR4-mediated Th2 cell chemotaxis is sensitive to the inhibition of PKC isoforms. Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells were preincubated with 10 μ M RO-32-0432 for 30 min. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing 100 nM MDC or 100 nM TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of RO-32-0432 (*, $p < 0.005$; **, $p < 0.0005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels. Levels of ERK1/2 and PKB phosphorylation in T-lymphoblasts were carried out by Western blot analysis. T-lymphoblasts (day 12-13) were washed three times in RPMI, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC or for indicated times, in the presence or absence of RO-32-0432 or 10 μ M Rottlerin (30 min preincubation), and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho ERK1/2 Ab or anti-phospho PKB⁴⁷³ Ab. The blots (B) were stripped and reprobed with pan-PKB to verify equal loading and efficiency of protein transfer. Results are representative of at least 2 separate experiments.

Figure 4.43. Both TLCK and TPCK are able to attenuate CCR4-mediated Th2 cell chemotaxis.

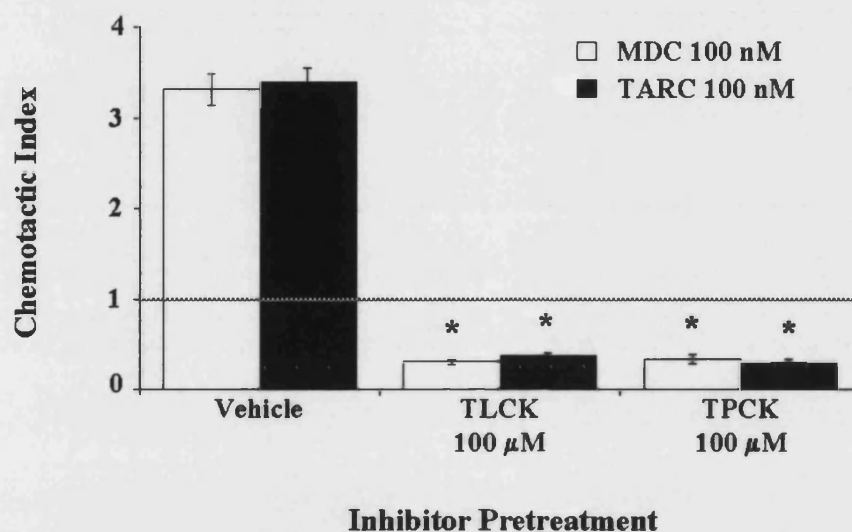


Figure 4.43. Both TLCK and TPCK are able to attenuate CCR4-mediated CEM cell chemotaxis. Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells were preincubated with TLCK or TPCK for 1 hour, at indicated concentrations. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing 100 nM MDC or 100 nM TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of two other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of inhibitor (*, $p < 0.0005$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Figure 4.44. JAK activity is not required for CCR4-mediated Th2 cell chemotaxis.

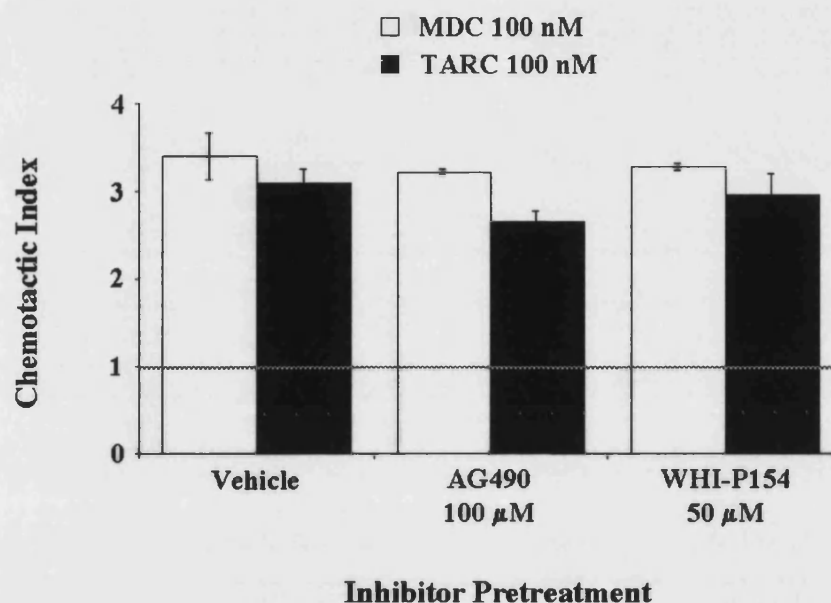


Figure 4.44. JAK activity is not required for CCR4-mediated Th2 cell chemotaxis.

Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells were preincubated with AG490 or WHI-P154 for 1 hour, at indicated concentrations. Cells (1×10^5 cells/25 μ l) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing 100 nM MDC or 100 nM TARC, as described in *Materials and Methods*. Chemotaxis across a 5 μ m membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of inhibitor ($p < 0.05$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Anti-Human MDC Treatment Potentiates MDC-mediated Th2 Cell Chemotaxis

The finding that pretreating Th2 cells with 10 µg/ml anti-human MDC Ab actually increased MDC-mediated chemotaxis was serendipitous (fig. 4.45). It was initially thought that the Ab being used was an anti-CCR4 Ab, being utilised to demonstrate that the TARC- and MDC-induced Th2 chemotactic responses were being mediated via CCR4. However, the alarming results of a 100% increase in chemotaxis for MDC, yet no effect on TARC responses, in the presence of anti-hMDC Ab soon led to the correct identity of the Ab.

MDC Stimulates the Phosphorylation of PYK2

PYK2 is a non-receptor tyrosine kinase structurally related to focal adhesion kinase (FAK) and largely has restricted expression to cells derived from the haematopoietic lineages (Avraham et al., 2000; Gelman, 2003). CXCL8, CCR5 and CXCR4 have demonstrated phosphorylation of PYK2 in response to their respective chemokine ligands and this tyrosine kinase has been implicated in having an important role to play in a cells ability to migrate (Davis et al., 1997; Di, V et al., 2004; Dikic et al., 1998; Fernandis et al., 2004; Ganju et al., 1998b; Ganju et al., 1998a). MDC stimulated the exceptionally rapid and transient tyrosine phosphorylation of PYK2, with phosphorylation occurring within and up to 1 minute post-stimulation (fig. 4.46, A). This phosphorylation was insensitive to PI3K, MEK and PKC inhibition (4.46, B).

MDC Induces the Phosphorylation of Both GSK-3 α/β and p90^{RSK}

The serine/threonine kinase p90^{RSK} is widely expressed and activated by ERK1/2, PDK-1 and PKC, with roles in cell cycle regulation, GSK-3 phosphorylation and regulation of gene expression via effects on transcriptional regulators such as NF- κ B (Frodin and Gammeltoft, 1999; Jensen et al., 1999). Two papers have confirmed p90^{RSK} phosphorylation can be stimulated by chemokines, namely CXCL12 and CCL5 (Lee et al., 2002; Zhang et al., 2002). MDC stimulated the phosphorylation of p90^{RSK}, with kinetics similar to that of

ERK1/2 phosphorylation (2 – 5 min), in CHOK1hCCR4 cells that was sensitive to PTX pretreatment (fig. 4.47, A).

GSK-3 is a multifunctional serine/threonine kinase involved in many different pathways and initially identified as an enzyme that regulates glycogen synthesis downstream of PKB (Doble and Woodgett, 2003; Frame and Cohen, 2001; Harwood and Braga, 2003; Harwood, 2001). However, only a single study has determined GSK-3 phosphorylation occurs downstream of a chemokine receptor, CXCL12/CXCR4 in a neuronal cell line (Chalasani et al., 2003). A recent study has suggested a role for GSK-3 in migration of astrocytes and has been implicated in controlling lamellipodia formation in keratinocytes (Etienne-Manneville and Hall, 2001; Koivisto et al., 2003). MDC stimulated the LY294002- and RO-32-0432-sensitive phosphorylation of GSK-3 α/β in T-lymphoblasts (fig. 4.47, B). PD98059 had no noticeable impact on this phosphorylation and LY294002 did not completely diminish the GSK-3 response (fig. 4.47, B).

Relocalisation of Arno-GFP in Response to MDC Stimulation

Arno (ARF nucleotide-binding-site opener) is a guanine-nucleotide exchange factor (GEF) for both ARF1 (ADP-ribosylation factor 1) and ARF6 *in vitro*, but specificity *in vivo* is likely to be more constrained to ARF6 (Macia et al., 2001; Turner and Brown, 2001). ARF6 targets include PLD and Rac1, with implicated roles in diapedesis of leukocytes, epithelial cell migration and receptor recycling (Claing et al., 2001; Donaldson, 2003; Powner and Wakelam, 2002; Santy and Casanova, 2001; Weber et al., 2001). The HUT-78 leukaemic T cell line (used because it is easier to transfect than CEMs) was transiently transfected with GFP-labelled Arno and stimulated with 10 nM MDC for time points up to 2 minutes. Reactions were stopped and cells fixed for analysis using a confocal microscope. Unstimulated cells displayed a punctate cytosolic localisation of Arno-GFP that, upon stimulation with MDC, became distinctly membrane localised at 1 min before returning to the cytosolic localisation, observed in unstimulated cells, at 2 minutes (fig. 4.48).

MDC and TARC Stimulate the Phosphorylation of UPE

CEM cells were stimulated with MDC and TARC over a time-course and cell lysates were generated for Western blot analysis. The blots were then immunoblotted for phosphorylated levels of a protein called FKHR (Forkhead in Rhabdomyosarcoma), a transcription factor activated downstream of PKB and involved in cell survival and regulation of the cell cycle. However, phospho-FKHR (MW 75 kDa; specific for Ser 256 phosphorylation) could not be consistently detected but instead an additional band at approximately 200 kDa was identified (fig. 4.49, A). This presence of this unidentified protein (termed 'Unidentified Phosphorylated Entity' or UPE) appears to be activation-induced and possibly shows phosphorylation between 5 and 10 minutes post-CCR4 ligation. This UPE phosphorylation was sensitive to PTX pretreatment of the CEM cells for both MDC and TARC-mediated responses (fig. 4.49, B).

Figure 4.45. 50% increase in MDC-induced Th2 cell chemotaxis on pretreatment of the cells with an anti-hMDC Ab.

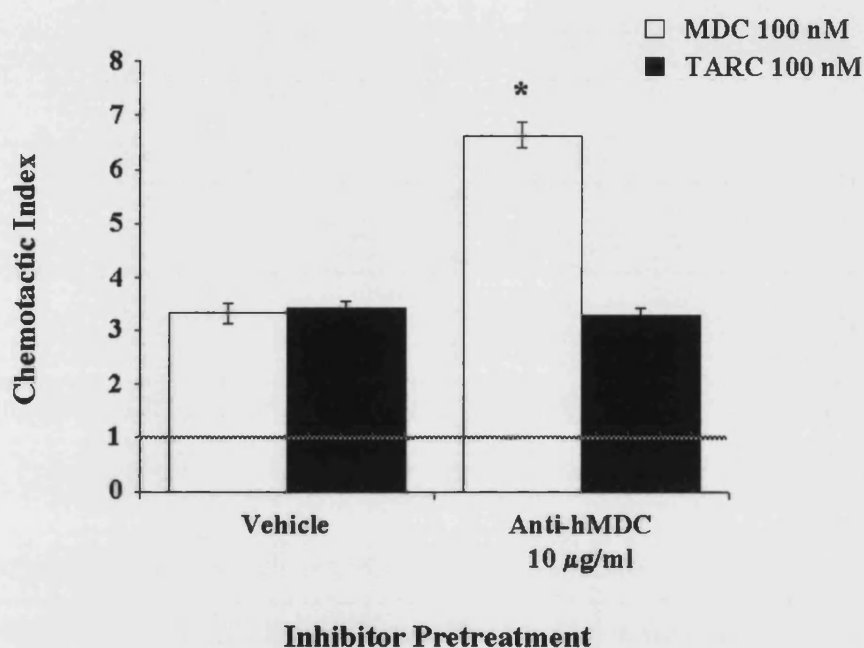


Figure 4.45. 50% increase in MDC-induced Th2 cell chemotaxis on pretreatment of the cells with an anti-hMDC Ab. Th2 cells were generated, as described in *Materials and Methods*, washed three times in RPMI, and resuspended in RPMI/0.1% BSA for 1 hour at 37°. Cells were preincubated with 10 µg/ml anti-hMDC Ab for 1 hour. Cells (1×10^5 cells/25 µl) were added to the upper wells of a disposable ChemoTx chemotaxis plate, above lower wells containing 100 nM MDC or 100 nM TARC, as described in *Materials and Methods*. Chemotaxis across a 5 µm membrane was determined after 1.25 hr incubation at 37°C in 5% CO₂. The data are derived from a single experiment with triplicate replicates that is representative of three other experiments. Data are expressed as the mean chemotactic index (\pm SEM), which is the ratio of cells migrating toward MDC vs. cells randomly migrating. Data were analysed by ANOVA and Student's *t* test with a Bonferroni correction to compare responses in the presence and absence of anti-hMDC Ab (*, $p < 0.001$). The horizontal line at a chemotactic index of 1 represents unstimulated basal migration and is shown for ease of comparison with stimulated levels.

Figure 4.46. CCR4 ligation with MDC leads to the phosphorylation of PYK2 in HUT-78 cells.

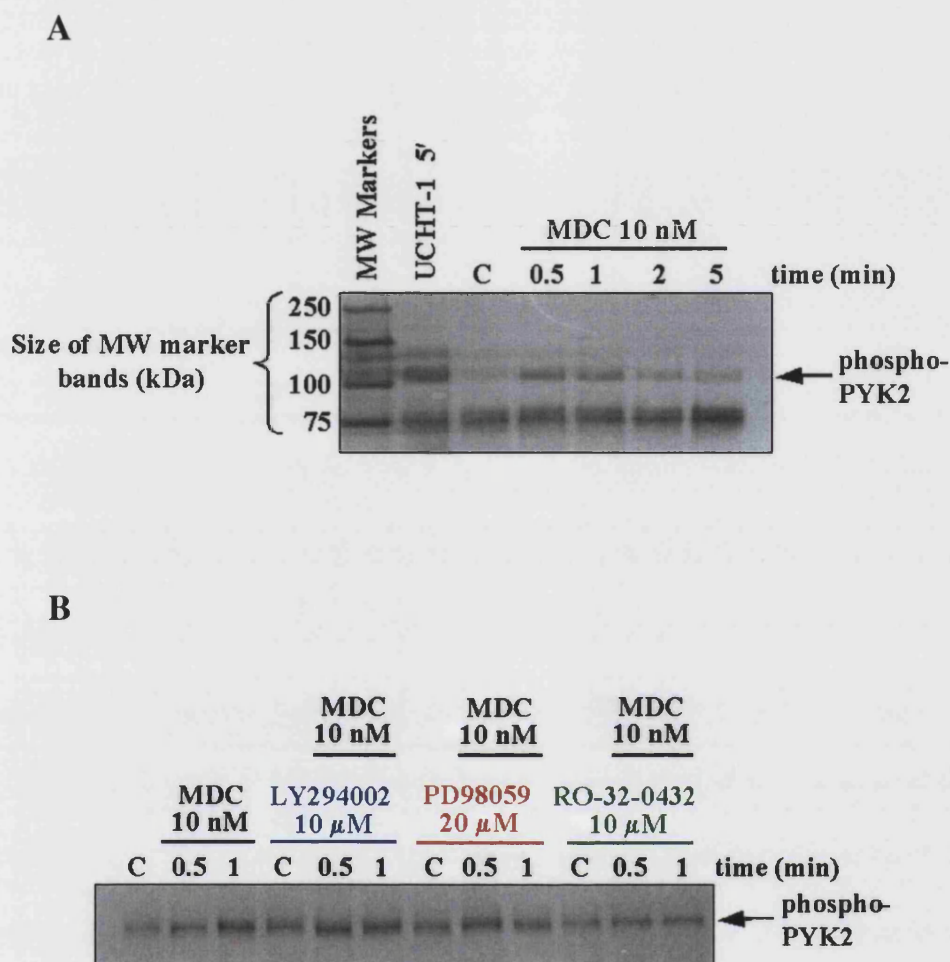


Figure 4.46. CCR4 ligation with MDC leads to the phosphorylation of PYK2 in HUT-78 cells. Levels of phospho-PYK2 protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in appropriate media, and resuspended at 4×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC or 10 μg/ml UCHT-1 (anti-CD3 Ab) for indicated times, *A*, and in the presence of indicated concentrations of LY294002 (30 min preincubation), PD98059 (60 min preincubation) or RO-32-0432 (30 min preincubation), *B*, and cells lysed by the addition of 500 μl ice-cold lysis buffer. PYK2 was then immunoprecipitated out by the addition of 3 μg/ml PYK2 Ab for 2 hours, followed by 20 μl of a 50:50 protein G slurry for 1 hour. Pelleted beads were then resuspended in 15 μl 2 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho tyrosine Ab (4G10 clone). Results are representative of at least 2 separate experiments (C = vehicle).

Figure 4.47. CCR4 ligation leads to the phosphorylation of both GSK-3 and p90^{RSK}.

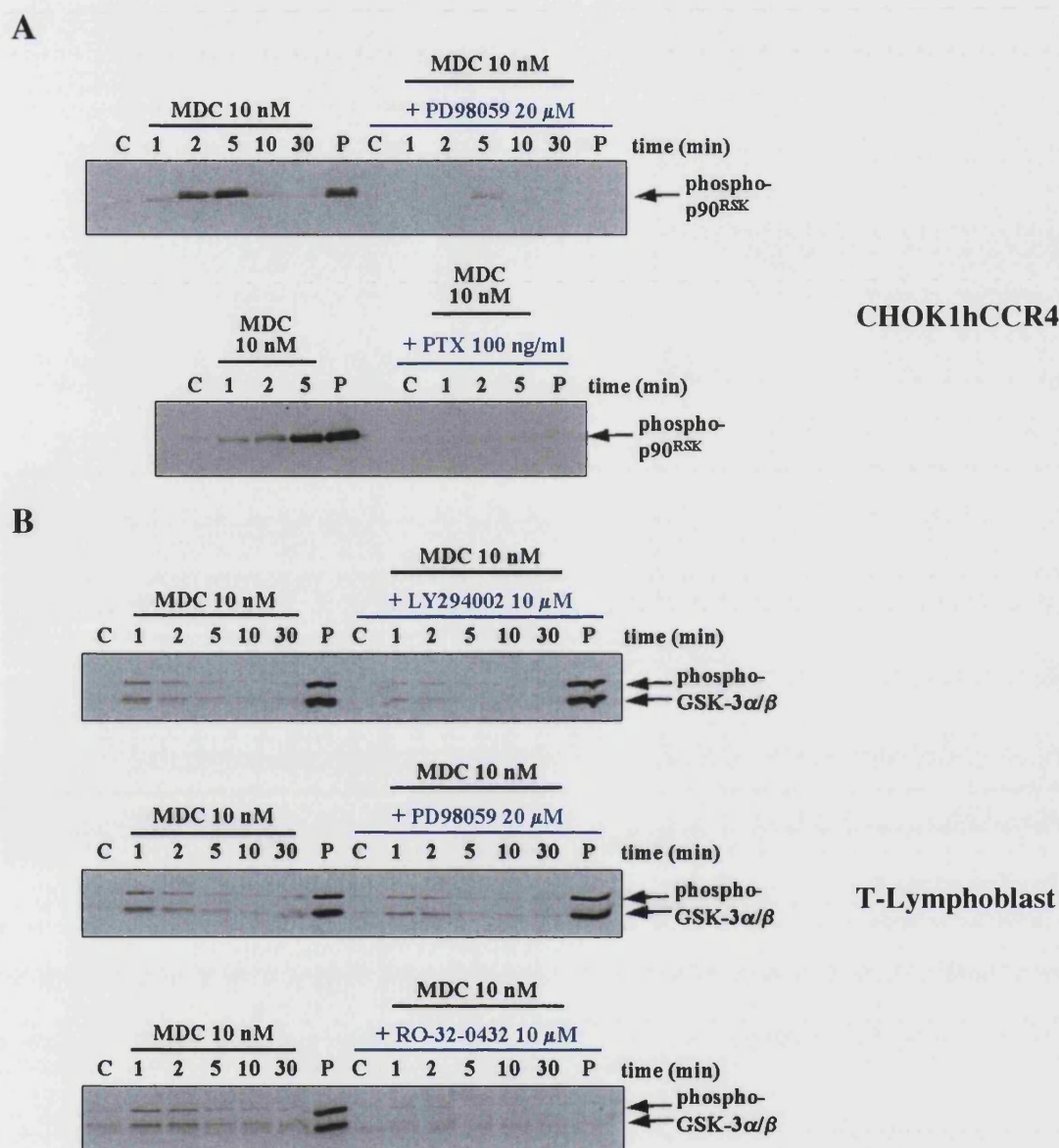


Figure 4.47. CCR4 ligation leads to the phosphorylation of both GSK-3 and p90^{RSK}. Levels of phospho-GSK-3 or -p90^{RSK} protein were determined by western blot analysis. Prior to experimentation, cells were washed three times in appropriate media, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. CHOK1hCCR4, A, or T-lymphoblasts, B, were then stimulated at 37°C with MDC for indicated times (in the presence or absence of indicated concentrations of LY294002, RO-32-0432 (both 30 min preincubation), PD98059 (60 min preincubation), or pertussis toxin (PTX, 16 hour preincubation) and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho GSK-3α/β Ab, with affinity for Ser 21 (of α) and Ser 9 (of β) phosphorylated forms, or anti-phospho p90^{RSK} Ab, with affinity for Ser 380-phosphorylated form. Results are representative of at least 2 separate experiments (C = vehicle; P = 100 nM PMA 5 min).

Figure 4.48. Localisation of Arno-GFP to the plasma membrane in HUT-78 cells upon stimulation with MDC.

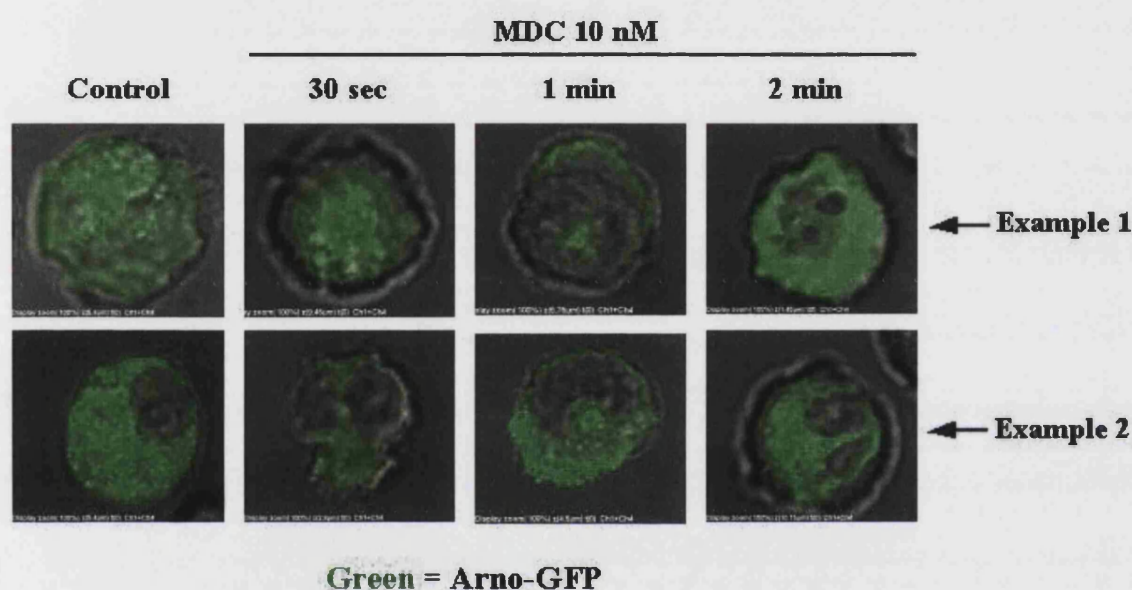


Figure 4.48. Localisation of Arno-GFP to the plasma membrane in HUT-78 cells upon stimulation with MDC. HUT-78 cells were transiently transfected with Arno-GFP and stimulated in a time-dependent manner with 10 nM MDC, with cells fixed in paraformaldehyde/glutaraldehyde. Images of the cells were obtained by confocal microscopy. Areas of green indicate localisation of Arno-GFP. Data are from a single experiment and representative of 2 separate experiments.

Figure 4.49. CCR4-ligation on CEM cells leads to the pertussis toxin-sensitive phosphorylation of UPE.

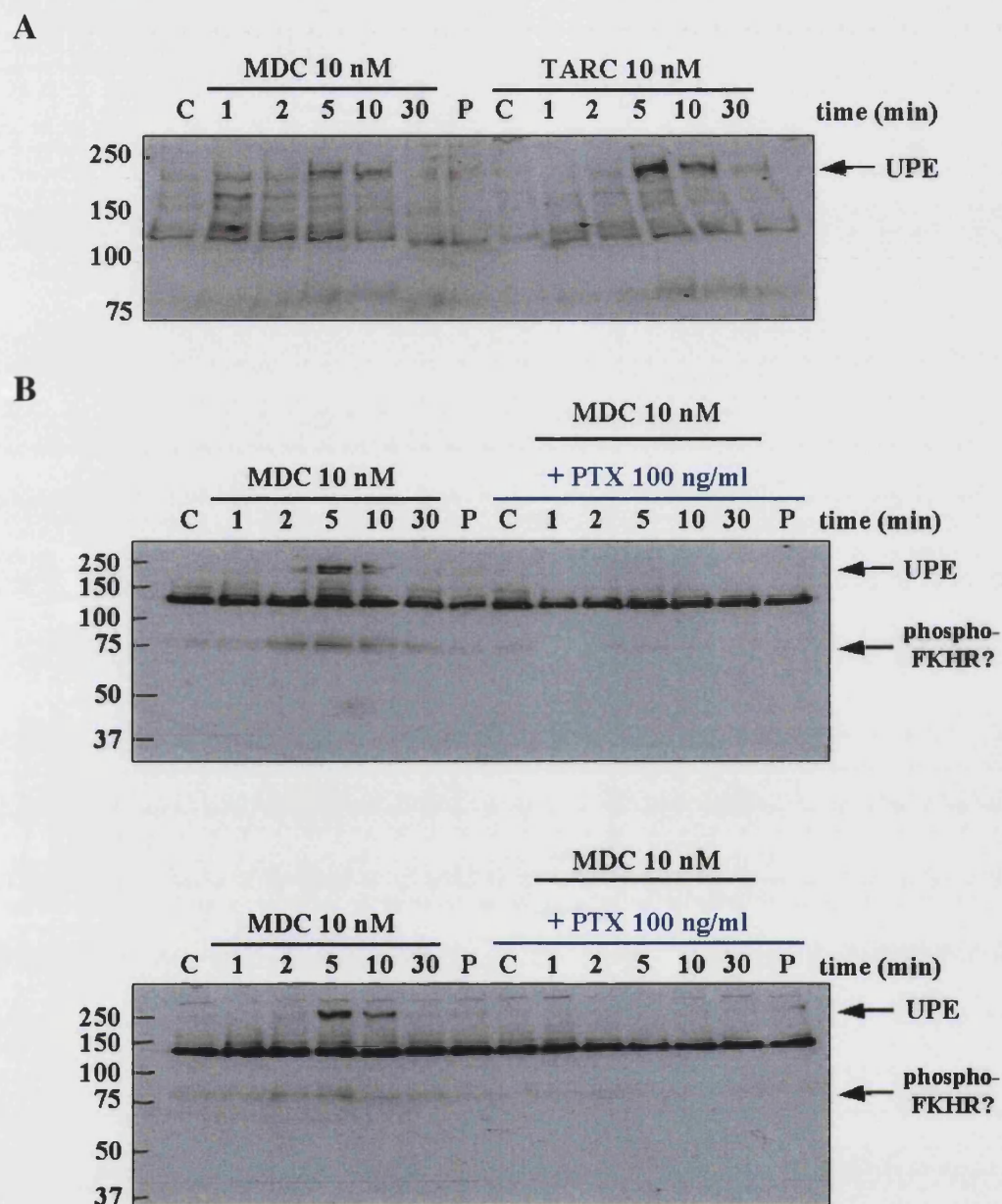


Figure 4.49. CCR4-ligation on CEM cells leads to the pertussis toxin-sensitive phosphorylation of UPE. Levels of phospho-‘Unidentified Phosphorylated Entity’ were determined by western blot analysis. Prior to experimentation, CEM cells were washed three times in appropriate media, and resuspended at 5×10^6 cells/ml and serum-starved for 1 hour. Cells were then stimulated at 37°C with MDC or TARC, *A*, for indicated times and in the presence or absence of 100 ng/ml pertussis toxin (PTX, 16 hour preincubation), *B*, and cells lysed by the addition of 1 x sample buffer. Cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti-phospho FKHR Ab, with affinity for the Ser 256-phosphorylated form of FKHR. Results are representative of at least 2 separate experiments (C = vehicle; P = 100 nM PMA 5 min).

Summary

- CCR4-ligation leads to intracellular calcium mobilisation in CHOK1hCCR4, CEM and Th2 cells.
- This calcium mobilisation requires $G\alpha_i$ and PLC activity, and IP3R calcium stores. Other sources of calcium, both intracellular and extracellular appear to contribute little towards this CCR4-induced calcium response.
- JAKs and PI3K have no significant role in these calcium responses, whilst caffeine has the ability to potentiate calcium flux. PKC activity has an unfavourable effect on calcium mobilisation.
- Th2 cells display similar responses as to those observed in CEM cells regarding intracellular calcium mobilisation and sensitivities towards pharmacological inhibitors.
- MDC and TARC stimulate the phosphorylation of ERK1/2 in a concentration- and time-dependent manner. With kinetics more rapid in the T cells than in CHOK1hCCR4.
- PKB and ERK phosphorylation, along with MDC-mediated CEM cell chemotaxis is PTX sensitive.
- Directed CEM cell migration induced by MDC is sensitive to PLC inhibition but refractory to the abolition of intracellular calcium mobilisation. ERK1/2 phosphorylation is partially sensitive to U73122 treatment.
- Classical PKC isoform inhibition has no impact on CCR4-mediated chemotaxis but broad-spectrum and PKC δ inhibitors have a severe detrimental response.
- MDC stimulates MLC2 phosphorylation, and abrogation of this effect with a ROCK inhibitor attenuates chemotaxis.
- TLCK and TPCK have harmful effect on PKB phosphorylation and chemotaxis in response to MDC stimulation. However, NOS, MEK/ERK, JAK and p38 appear to be redundant for CCR4-mediated CEM cell chemotaxis.
- Data from Th2 cell migration in response to MDC correlated exceptionally well with data obtained from CEM cells.

- Anti-human MDC Ab potentiates Th2 cell migration in response to MDC but not TARC.
- CCR4 ligation leads to the phosphorylation of PYK2, GSK-3 α/β and p90^{RSK}.
- MDC stimulates the membrane localisation of Arno-GFP in HUT-78 cells and the phosphorylation of a ~ 200 kDa mystery protein.

4.3 Discussion II

CCR4-Mediated Intracellular Calcium Mobilisation

The rise in intracellular calcium represents a common activation pathway upon triggering cells with chemokine ligands. However, the mechanisms that lead to this release of calcium are poorly understood. There have been a number of advances in the last few years within the calcium field itself, with the discovery of sphingosine-, NAADP- and cADPR-mediated calcium release mechanisms, and it appears that these pathways are able to overlap considerably with the more established IP3R, RR and extracellular influx routes. This has led to many complex models being proposed on how the various mechanisms may be intertwined based on studies performed in a variety of systems. In contrast, chemokine-induced calcium mobilisation is largely used as an indicator of agonistic activity for chemokines upon exposure to various cell types, with very little interest/research into the underlying mechanisms. Unsurprisingly, the results presented within demonstrated the rapid and transient mobilisation of calcium in CHOK1hCCR4, CEM and Th2 cells in response to CCR4 ligation. MDC and TARC have previously been shown to instigate a calcium flux in Th2 cells (Andrew et al., 1998; D'Ambrosio et al., 1998), NK cells (Inngjerdingen et al., 2000), and various CCR4 transfected cell lines (Imai et al., 1997; Imai et al., 1998). The kinetics for these studies varies from 30 seconds up to ~100 seconds for the span of time that the calcium concentration is above resting levels. The Th2 studies cited above, however, are very comparable to the results presented here for both CEM and Th2 cell calcium responses (about 90 seconds). The more rapid 'on-off' calcium responses observed for CHOK1hCCR4 cells is possibly due to the differences in technique employed and to cell type. MDC was consistently more effective than TARC in eliciting calcium mobilisation and this is consistent with their respective abilities in phosphorylating/activating other signalling mediators, such as PKB and ERK.

Role of IP3R Calcium Stores

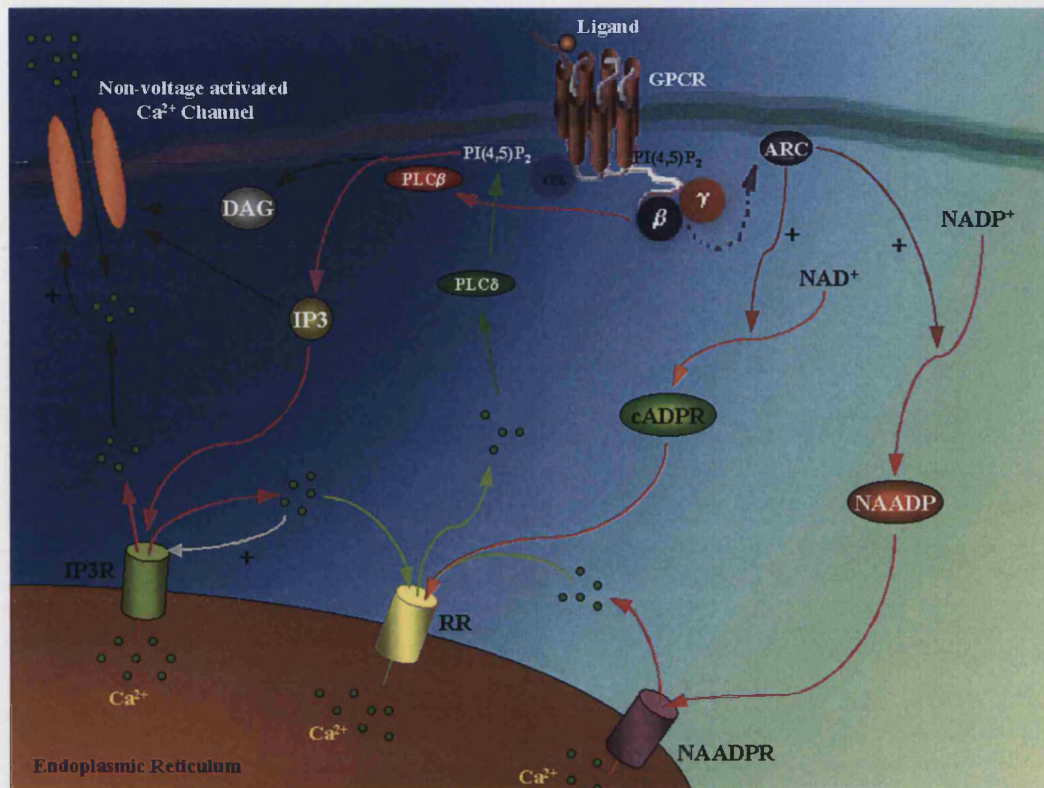


Figure 4.50. Schematic showing the potential chemokine-activated calcium mobilising pathways. Note that not all possible pathways are displayed in order to minimize the complexity. Other pathways include the sphingosine-1-phosphate cascade that has been demonstrated to be activated downstream of GPCRs. Abbreviations not defined in text: ARC, ADP-ribosyl cyclases.

As is the case for the vast majority of chemokine-mediated responses, PTX treatment had the effect of abolishing the chemokine-mediated calcium flux in both CEM and Th2 cells, indicating that CCR4 is coupled to the G α i protein (Schorr et al., 1999). Although PTX fails to distinguish between α i- and β γ -stimulated pathways, it is likely that the β γ subunits are responsible for eliciting the calcium response observed with MDC and TARC. The PLC inhibitor U73122 completely attenuated the CCR4-mediated calcium response but α i has never been shown to couple to PLC β , whereas the β γ subunits are known to be capable of stimulating the PLC β $_2$ and β $_3$ isoforms that are activated by chemokine receptors (Jiang et al., 1997; Li et al., 2000; Rhee and Bae, 1997; Wing et al., 2001; Wu et al., 1993).

Where investigated, it is evident that the majority of chemokine-mediated calcium responses are wholly-sensitive to PLC inhibition, thereby indicating a requirement for IP₃-mediated calcium release (Maghazachi, 1997; Scandella et al., 2003; Schorr et al., 1999; Soriano et al., 2003). However, this inhibition does not confirm that IP₃R calcium stores are the sole contributors to the calcium response, and merely that they are essential for the instigation of a calcium flux. Figure 4.50 is a simplified diagram of some of the interactions that occur between the different calcium pools.

Role of cADPR-Mediated Calcium Mobilisation

Indeed, it has been demonstrated that CXCL12 may utilise the cADPR pathway in NK cells in addition to the IP₃R cascade, and the same study suggested that MDC- (and CXCL8) mediated intracellular calcium mobilisation was solely via the cADPR pathway and that the IP₃R pools contributed very little to the overall calcium response (Inngjerdingen et al., 1999). Treating the cells with an anti-RR Ab abolished this MDC-mediated calcium flux suggesting that cADPR is acting via the RR stores, as would be expected. The mechanism surrounding cADPR-mediated responses are still very unclear. It is known that cADPR represents more of a modulator rather than a messenger as its introduction into a cell will normally have no immediate effect, suggesting that it functions by increasing the sensitivity of RRs to rises in calcium, propagating a CICR effect (Cui et al., 1999; Empson and Galione, 1997; Hashii et al., 2000). How cADPR does this is unknown. One proposal is that it binds directly to RRs and stimulates calcium release, although cADPR appears not to bind directly to the RR and likely functions through an intermediary protein, such as the FKBP12.6 (FK506-binding protein 12.6) that does associate with RRs (Lee, 1997; Noguchi et al., 1997). Another recent suggestion is that it activates the SERCA pump thereby enhancing the calcium load within the ER lumen – a process that is known to increase the sensitivity of RRs (Koizumi et al., 1999; Lukyanenko et al., 2001). In contrast to the Inngjerdingen et al. study, inhibition of the cADPR pathway and RRs had no appreciable effect on CCR4-mediated calcium release in CEM cells. This maybe down to differences in cell type utilised between the two studies. Inngjerdingen et al. showed that there was a relatively large delay (~ 30 sec) between the addition of MDC and the mobilisation of calcium. This is in contrast to other studies, in various cell types, observing CCR4-

mediated calcium mobilisation that show an immediate effect of the ligand in producing a calcium flux. The technique employed by Inngjerdingen also required the permeabilisation of the cells in order to treat with various inhibitory tools and this may have had negative consequences upon the signalling mechanisms. The same group suggest that the stimulation of this pathway is via the G α s protein (not α i) (Maghazachi, 2000). This is clearly contradictory to the results presented within this thesis. However, a recent paper has suggested a role of CD38, an ADP-ribosyl cyclase, in chemokine-stimulated DC calcium mobilisation and chemotaxis (Partida-Sanchez et al., 2004).

NAADP Does not appear to Contribute to CCR4-Induced Calcium Responses

In contrast to IP3Rs and RRs, the NAADP release mechanism is not sensitive to calcium and therefore does not support CICR. To date, there have been no studies into the role of this pathway in chemokine-mediated calcium mobilisation. This is likely due to the fact that there is a distinct lack of tools to investigate this pathway. The pharmacological inhibitors that do exist are not specific to the NAADPR, and all were developed, and are effective, as L-type calcium channel inhibitors (Patel et al., 2001). It is proposed that NAADP may function to sensitise the IP3Rs and RRs by providing a small amount of calcium that triggers the receptors, or by releasing a bolus of calcium that is taken up by other stores in the lumen and sensitises the IP3R/RRs this way (Berridge et al., 2003). Nifedipine, an L-type calcium channel blocker, has been proven to inhibit NAADP-mediated calcium release but had no effect on MDC- or TARC-induced calcium responses (Yusufi et al., 2002). This suggests there is no role of this cascade in the response, which is further backed up by the complete inhibition observed with 2-APB and U73122, which would not be expected if NAADP were contributing to the calcium flux as it appears to not lie downstream of the IP3 cascade but parallel.

RR Involvement Downstream of Ligated CCR4

Inhibition of RRs with dantrolene and inhibitory concentrations of ryanodine had no impact on CCR4-mediated CEM cell calcium mobilisation. However, caffeine surprisingly potentiated the CCR4-stimulated calcium mobilisation whilst having no effect on its own.

The RR possesses calcium-binding high-affinity activation and low-affinity inhibition sites with competition between the two. A full-blown calcium release by caffeine requires a small calcium release, initiated by caffeine itself, which then acts on RRs triggering a large calcium release (Islam, 2002). The second step of this can be inhibited if the initial small rise in calcium is mopped up by calcium-binding proteins, such as calreticulin and calsequestrin, that are abundant in leukocytes (Pettit and Fay, 1998). Because caffeine was incubated with the cells for 30 min prior to analysis it is likely that any calcium response generated upon initial exposure to caffeine had been adequately buffered so as not to display high basal levels of cytosolic calcium, but caffeine was unable to stimulate the release of calcium when it was added during analysis. Caffeine may still have been having the effect of increasing the calcium affinity of the calcium activation site of RR so that the calcium released by IP3R stores is activating the RRs and subsequently amplifying the CCR4-mediated calcium response. This would also explain the ability of ryanodine in inhibiting this effect with no ramifications on the non-caffeine influenced response.

Role of JAKs, PI3K and PKC in Intracellular Calcium Mobilisation

A couple of groups have published a number of papers implicating a role of the Janus kinases in chemokine-mediated signal transduction. The JAKs are a well-known signalling intermediary in cytokine-induced signal transduction; however, their role in chemokine signalling is controversial. It has been postulated that JAKs lie upstream of everything in the signal cascade elicited by chemokines, and that by inhibiting JAKs, by definition, activation of all other pathways are similarly inhibited (Mellado et al., 1998; Rodriguez-Frade et al., 1999a; Soriano et al., 2003). These studies demonstrate abrogation of JAK-activated downstream effectors with 50 μ M AG490. Published studies have focused mainly on CCR2-, CCR5- and CXCR4-mediated responses in monocytes and T cells, respectively. However, even at 100 μ M, pretreatment of CEM or Th2 cells with AG490 was far from abolishing the CCR4-mediated calcium flux, whilst JAK3 inhibition (also implicated in some studies) alone had no effect whatsoever. This was also the case for CCR8 T cell migration and calcium flux (Wain and Ward, unpublished data). Additionally, another study has refuted a role for JAKs in CXCR4-mediated signalling (Majka et al., 2000a). Thelen and Baggiolini (Thelen and Baggiolini, 2001) proposed a number of

reasons why receptor dimerization and the JAK-STAT pathway are unlikely to be functionally relevant for chemokine receptors. One of the more appealing arguments against involvement of the JAK-STAT pathway are the kinetics of calcium mobilisation. Chemokines initiate a calcium response within 1 sec of their addition to the cellular environment, but cytokines have a 20 – 30 sec delay. This is likely due to the time required for receptor oligomerisation to occur (involving plasma membrane movement) and then subsequent activation of JAKs, G-proteins and PLC isoforms, etc. Therefore, it would be expected that if chemokines were utilising this JAK mechanism that the kinetics for calcium mobilisation would be more delayed than previously shown, which is not the case. Additionally, the vast majority of calcium responses are PTX-dependent, and with respect to certain receptors, such as CCR4, all responses investigated have been demonstrated to be PTX sensitive. There seems little benefit from having an additional signalling entity that solely recruits PTX-sensitive G-proteins. It has been suggested by Mellado et al. that combination of chemokines (i.e. CCL2/CCR2 and CCL5/CCR5) switch the calcium flux response from PTX-sensitive to insensitive. Indeed, in many disease situations there are multiple chemokines released at a similar time and this may present a more plausible explanation for the need of receptor dimerization-mediated JAK signalling. Differences in the requirement for JAKs may be explained in part by the use of different cell types with most of the CXCR4 T cell work done in MOLT-4 cells (a human acute T lymphoblastoid leukaemia cell line). However, all migratory events to date have been sensitive to PTX treatment, indicating that any PTX-insensitive events are superfluous for chemotaxis. This area needs to be more intensely investigated by more groups (currently 3 groups have produced the vast majority of the published work, with only ~10 primary papers published in this area in total) before a more balanced judgement can be formulated. The use of phospho-specific Abs against JAKs will help to determine if they are downstream of CCR4.

PI3K has been shown to be pivotal in the CX3CR1-mediated calcium response in transfected CHO cells, with wortmannin and LY294002 completely attenuating any CX3CL1-induced calcium response, whilst PLC inhibition had no effect (Kansra et al., 2001). This is thought to occur through an unknown receptor-operated channel. PI3K is known to have an excitatory role on L- and N-type calcium channels (voltage-operated calcium channels) via the $\beta\gamma$ subunits, but blockade of these channels had no effect on the

influx of calcium (Blair and Marshall, 1997; Viard et al., 1999). Besides, leukocytes are not excitatory cells and thus not voltage-activated, therefore discounting any effect of N- or L-type calcium channels in a chemokine-mediated calcium response in T cells. This is confirmed with the lack of effect of nifedipine (an L-type channel blocker) on CCR4-induced CEM cell calcium mobilisation. It is generally considered that calcium influx pathways contribute little, if at all, to the immediate chemokine-mediated calcium responses, but calcium release-activated calcium current (CRAC) channels may have a part to play in sustaining an elevated calcium level (Schondorf et al., 1993; Schorr et al., 1999). These channels are activated due to intracellular calcium store depletion in response to, for example, IP3 activity on IP3R (these channels are also called store-operated channels (SOCs)). Some of these SOCs (along with some ROCs and other channel types) form part of the transient receptor potential (TRP) gene superfamily of calcium permeable cation channels and thought to be the most promising molecular candidates for the otherwise unknown leukocyte calcium influx channels (Li et al., 2002). These TRP channels have been demonstrated to be activated by DAG in addition to PI3K and other mediators (Gamberucci et al., 2002). However, PMA treatment had a detrimental impact on calcium responses initiated by MDC or TARC in CEM cells and LY294002 treatment had no influence on calcium flux. In addition, the inhibition of PKC increased the intracellular mobilisation of calcium further opposing the possibility of TRP channel activity in CCR4-stimulated calcium responses in T cells. It is highly likely that this effect of PKC on calcium response is due to effects on receptor internalisation (Guinamard et al., 1999; Schondorf et al., 1993). It has been demonstrated that phorbol esters stimulate the phosphorylation of CXCR4 and that this leads to the rapid internalisation of the receptor that can be blocked by PKC inhibitors (Haribabu et al., 1997; Signoret et al., 1997). The potentiation in calcium response observed with PKC inhibitors on MDC/TARC-responses is likely due to the inhibition of ligand-dependent phosphorylation of CCR4 leading to internalisation via the activation of PKC. However, experiments need to be conducted to confirm the downregulation of CCR4-receptor expression in response to PMA and MDC/TARC and the reversal of this phenomenon with PKC inhibitors.

Overall, the MDC- or TARC-mediated calcium response is a reasonably rapid 'on-off' affair, suggesting that there is little, if any, role of a calcium influx mechanism in a CCR4-

induced calcium flux in T cells. It would appear that the majority of the response is mediated via IP3R stores, with no contribution from other intracellular calcium release mechanisms. However, more thorough investigations into the role of TRPs, and other influx channels, needs to be conducted for chemokine-elicited calcium responses to be able to draw more conclusive deductions about the role of calcium influx pathways.

CCR4-Induced T Cell Migration

How a Cell Crawls

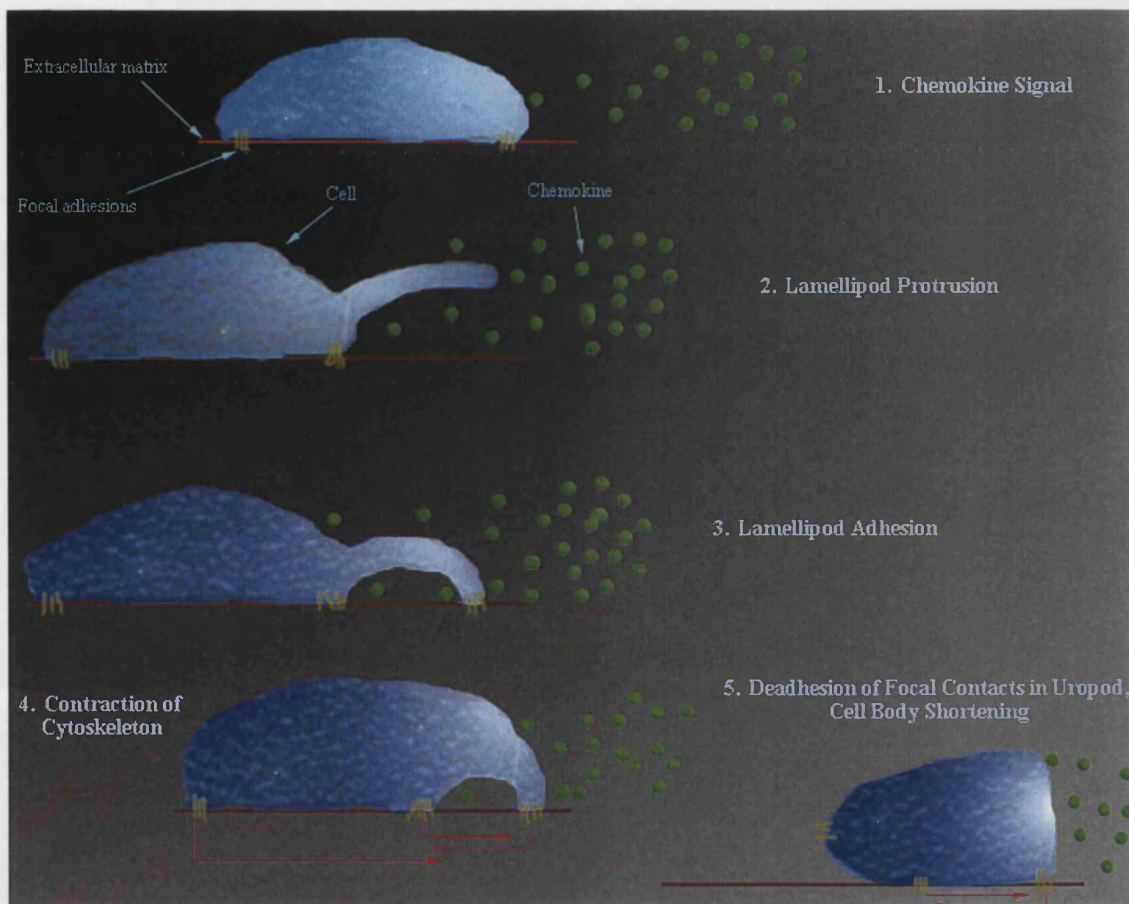


Figure 4.51. Model of cell locomotion. 1). A resting cell detects a chemoattractant gradient and becomes activated. 2). The cell protrudes a lamellipod towards the source of the chemokine. 3). New focal contacts (mediated by integrins) are formed under the extended lamellipod. 4). The cell exerts force on the cytoskeleton via contraction (direction of force is displayed by red and pink arrows). 5). The old focal contacts at the rear of the cell detach under the exerted force, resulting in net translocation of the cell.

Cell locomotion can be considered as a process that involves 5 steps, with the overall process appearing as a crawling movement. This process requires cycles of actin polymerisation and depolymerisation. Upon a resting cell being stimulated by a chemokine, the signal generated leads to the amplification of barbed ends of actin (known as microspikes or filopodia) that elongate to form filaments (lamellipodia) that can protrude many micrometres long from the cell body at the leading edge. This protruding edge then adheres to the substratum (extracellular matrix) via focal complexes, followed by development of contractile force on the cytoskeleton due to the contraction of the cytoplasmic actomyosin. The old focal contacts at the trailing edge (uropod) of the cell detach, resulting in the cell's net translocation (fig. 4.51). In general, cdc42 regulates filopodia, Rac is required for lamellipodia formation and Rho is involved in the control of cell contraction. However, the identification of the pathways that lie upstream and downstream of these small GTPases, and any cross talk that occurs between them, are largely unknown and appear to vary greatly depending on the experimental system. This section will try to identify signalling mediators and propose pathways that may be required for CCR4-induced T cell chemotaxis.

Calcium Mobilisation is not required for CCR4-Mediated T Cell Chemotaxis

As has previously been discussed, the vast majority of chemokine-mediated events are PTX-sensitive, with all investigated migratory events sensitive. CCR4-mediated actions produced no surprises in this area with ERK1/2, PKB, p90^{RSK} and UPE phosphorylation dependent upon G α i coupling to CCR4, in addition to CEM and Th2 cell chemotaxis.

There have been many roles for calcium in cell motility and polarity proposed, from the regulation of actin-binding proteins to the stimulation of myosin II-based contraction (Stossel, 1993). MLCK activates myosin II, which provides the force necessary for lamellipod extension and uropod retraction, and calcium/CaM binding directly activates MLCK. Although chemokines have been demonstrated to mobilise calcium, the requirement of a measurable calcium flux for chemokine-mediated cell migration remains ambiguous. It seems likely that the need for calcium mobilisation for migration is dependent upon the type of cell and stimulating chemokine. In the giant newt eosinophil

and *Dictyostelium* cells, upon stimulation there is an initial calcium release from the central store caused by IP₃, followed by a fall in calcium concentration in the part of the cell nearest the chemoattractant source. This generates a small calcium gradient and probably contributes towards the cell's polarisation (Brundage et al., 1991; Brundage et al., 1993; Gilbert et al., 1994; Yumura et al., 1996). A similar gradient in the distribution of calcium/CaM has also been demonstrated in the fibroblast (Hahn et al., 1992). A measurable calcium gradient has been difficult to demonstrate or is absent in smaller cell types such as human neutrophils (Laffafian and Hallett, 1995). However, some studies report that transient increases in calcium mobilisation are required for chemotaxis in these cell types, such as CXCR4- (haemopoietic stem cells) and CCR7- (DCs) mediated migration (Henschler et al., 2003; Scandella et al., 2003). Whilst other studies suggest that chemotaxis does not rely upon a calcium flux, such as CCR2 in monocytes (Turner et al., 1995). Indeed, calcium mobilisation appears to be inconsequential for CCR4-mediated T cell chemotaxis, as results demonstrate in this thesis with the lack of effect of 2-APB on migration, yet the compound inhibits calcium flux. Although MLCK can be activated by a calcium/CaM-dependent mechanism, its activation has also been demonstrated to occur via the Rho-ROCK pathway (Vicente-Manzanares et al., 2002).

Roles of PKC and ROCK for Directed T Cell Migration in Response to CCR4 Ligands

Cell migration is a complex process with multiple signalling pathways likely to be required for a cell to assume a characteristic polarised morphology, extend membrane processes (lamellipodia) at the leading edge of the cell, and then pull the rear of the cell forward. Rho is a small GTPase whose activity has been implicated heavily in this cell polarisation process. The first effector of Rho to be discovered was the Rho-associated coiled-coil forming protein kinase (ROCK) II (also known as ROK α), with another family member, ROCKI (ROK β), since discovered (Ishizaki et al., 1996; Leung et al., 1995; Leung et al., 1996; Matsui et al., 1996; Nakagawa et al., 1996). ROCKs phosphorylate various substrates, such as myosin light chain (MLC) phosphatase (MLCP), MLC and LIM (Lin11, Isl1 and Mec3) kinase (LIMK). ROCKs are able to phosphorylate MLC, independently of calcium, on the same residue that is phosphorylated by MLCK (Ser 19) (Amano et al., 1996; Totsukawa et al., 2000). This phosphorylation of the regulatory MLC (also known as

MLC2) of myosin II induces its interaction with actin, leading to activation of myosin ATPase and the subsequent enhancement of cell contractility (required for uropod detachment). MLCP is responsible for the dephosphorylation of MLC, and so negatively regulates actomyosin-based contractility. ROCK phosphorylates the myosin-binding subunit (MBS) of MLCP at Thr 697 (inhibitory), Ser 854 and Thr 855 (phosphorylation of this particular residue leads to MLCP dissociating from myosin), but the exact mechanisms of inhibition are not known (Feng et al., 1999; Kawano et al., 1999; Kimura et al., 1996; Velasco et al., 2002). Another ROCK substrate are the serine/threonine LIMKs that then phosphorylate and inhibit cofilin. Cofilin is an actin-binding and -depolymerising protein that regulates the turnover of actin filaments, thus inhibition leads to an increase in the number of actin filaments (Maekawa et al., 1999). Despite this evidence for a role of ROCK in cell motility there have only been a couple of papers published concerning the role of ROCK in chemokine-mediated directed cell migration. In both studies a pharmacological inhibitor (Y-27632) of ROCKI and II was used to ascertain its importance in chemotaxis. CXCR4- and CCR7-mediated chemotaxis of T lymphocytes was abrogated with the inhibitor (Bardi et al., 2003; Vicente-Manzanares et al., 2002) and Vicente-Manzanares demonstrated that MLC phosphorylation in response to CXCL12 was dependent upon RhoA. CEM cell chemotaxis in response to MDC displayed a similar sensitivity towards ROCK inhibition with Y-27632. Furthermore, MDC stimulated an increase in the phosphorylation of MLC that was abated with Y-27632 pretreatment. Interestingly, the inhibition of MLC phosphorylation was complete and thus well below the basal levels. This would suggest that ROCK is doing more than just inhibiting MLCP, which is regarded as the most likely mechanism of action for ROCK in actin polymerisation, and must be playing a proactive role in the phosphorylation of MLC, either directly or possibly indirectly via MLCK (Vicente-Manzanares et al., 2002).

Although the G protein $\beta\gamma$ subunits have been demonstrated to activate various Rho guanine nucleotide exchange factors (GEFs), including p114RhoGEF (Niu et al., 2003), and thus activating the Rho-ROCK pathway, recent evidence provides a link for PKC in MLC phosphorylation, independent of activating Rho. Results presented within have established that PLC is critical for CCR4-mediated chemotaxis (a phenomenon displayed by other chemokine receptors as well (Smit et al., 2003; Soriano et al., 2003)). Having

determined that calcium mobilisation seems not to be required for CCR4-mediated migration, it would therefore appear that the second product of PLC-mediated PI(4,5)P₂ hydrolysis, namely DAG, has a pivotal role to play. A recently published paper, utilising oligodeoxyribonucleotides (ODN) to specifically inhibit PKC β and various pharmacological inhibitors, provided evidence that PKC β was required for monocyte chemotaxis in response to CCL2 (Carnevale and Cathcart, 2003). In addition, PKC ζ was shown to be essential for neutrophil chemotaxis in response to the chemokine CXCL8 (Laudanna et al., 1998). However, PKC β is a classical PKC isoform that requires both DAG and calcium for its activity and would thus suggest it is an unlikely candidate as a PKC isoform in CEM cell migration towards MDC. This is confirmed with the lack of effect of the classical PKC inhibitor Gö6976 on this migratory event. Since DAG appears to be involved it is possible that a novel PKC isoform maybe mediating CEM cell chemotaxis, although a role for PKC ζ in CCR4-mediated CEM cell migration cannot be ruled out. This conclusion would seem to be confirmed with the sensitivity of MDC-induced T cell migration being sensitive to the broad-spectrum (classical and novel isoform) PKC inhibitor RO-32-0432. It is not understood how PKC isoforms regulate cell motility/migration. One hypothesis maybe that PKC is having effects on actin reorganisation/polymerisation. Effectors of PKC isoforms are the MARCKS (myristoylated alanine-rich C kinase substrate) family of proteins (Arbuzova et al., 2002). It has been shown that MARCKS can bind to and cross-link actin *in vitro* and that activation of the PKC pathway leads to the local release of actin by MARCKS, resulting in a local softening of the actin cytoskeleton and increased plasticity (favourable for migration through tight spaces) (Hartwig et al., 1992). MARCKS has also been found to co-localise with F (filamentous)-actin in the lamellipodia (Myat et al., 1997). This regulation of actin by MARCKS may alternatively occur via PI(4,5)P₂ availability. MARCKS has been shown to sequester PI(4,5)P₂ and this lipid may be released by PKC activity allowing the lipid to become locally available for actin-binding proteins, a hypothesis that is supported by the fact that MARCKS can inhibit PLC-induced PI(4,5)P₂ hydrolysis and this can be reversed by PKC (Wang et al., 2001). Alternatively, PKC may be involved in the phosphorylation of MLC. The protein kinase C-potentiated inhibitor protein of 17 kDa (CPI-17) specifically inhibits MLCP when it is phosphorylated at Thr 38. A very recent study established that all PKC isoforms were able to interact with CPI-17 *in vivo*, via their

cysteine-rich domains, and that they were responsible for the phosphorylation of the Thr 38 residue of CPI-17 (Zemlickova et al., 2004). Additionally, PKC has been shown to be required for the activity of ROCK, without affecting Rho-ROCK complex formation (Barandier et al., 2003). Moreover, EGFR-mediated myosin heavy chain A and B (MHC-A, -B) phosphorylation is dependent upon PKC activity (Straussman et al., 2001), and EGF-induced MLC phosphorylation required PKC δ (myosin II is a hexamer composed of two heavy chains and two pairs of light chains) (Iwabu et al., 2004). Incidentally, although PDK-1 is the upstream kinase for the activation loop of PKC isoforms, it has been reported that PDK-1 can mediate this phosphorylation event independent of PI3K and Iwabu et al. present data indicating that PI3K inhibition had no effect on PKC δ activity (Dempsey et al., 2000; Iwabu et al., 2004; Sonnenburg et al., 2001). The importance of the PKC δ isoform was highlighted within this thesis with the observation that the PKC δ selective inhibitor rottlerin completely attenuated CEM cell chemotaxis in response to MDC. This was in accordance with another study that demonstrated that CCL15/CCR1 chemotaxis relied upon PKC δ activation (Ko et al., 2002). Rottlerin is a controversial inhibitor as it has been reported to display no inhibitory activity against PKC δ whatsoever (Davies et al., 2000), whilst other groups have contradicted this data (Saraiva et al., 2003). Finally, PKC δ has been shown to activate NF- κ B in neutrophils and epithelial cells (Page et al., 2003; Vancurova et al., 2001), and NF- κ B activity is required for CCL15/CCR1- and CXCL12/CXCR4-mediated chemotaxis (Cherla and Ganju, 2001; Ko et al., 2002). Activation and nuclear translocation of NF- κ B requires phosphorylation, ubiquitination, and subsequent degradation of its cytoplasmic inhibitor I κ B α via the I κ B kinase (IKK) pathway. Both TLCK and TPCK have been reported to prevent the degradation of I κ B α and thus preventing NF- κ B activation (Henkel et al., 1993; Zerneck et al., 2001). TLCK and TPCK treatment of CEM cells abolished their migratory response towards MDC. Although these inhibitors are broad-spectrum protease inhibitors they give an idea as to the requirement of NF- κ B in cell migration as demonstrated by other groups that have then gone onto use more selective NF- κ B inhibitory methods (Cherla and Ganju, 2001). It is not known how NF- κ B is contributing towards the chemotactic response. Cherla et al. propose that it is via the NOS pathway, with NOS inhibition detrimental to CXCL12-induced T cell chemotaxis, but pretreatment with L-NAME had no effect on CCR4/CEM cell chemotaxis (Cherla and Ganju, 2001). Therefore, it is more likely reliant upon the transcription of new

genes and subsequent translation into proteins. This hypothesis is supported by the detrimental effects of actinomycin D (RNA synthesis inhibitor (prevents RNA polymerase moving along DNA – transcription) and cyclohexamide (protein synthesis inhibitor - translation) on CCL15-induced cell migration (Ko et al., 2002).

However, it is possible that the DAG product of PI(4,5)P₂ hydrolysis is responsible for mediating migration by more than just activation of the PKC pathway. The last few years have seen an increasing amount of data on the additional signalling pathways promoted by DAG. One of these pathways is the activation of Rap-GEFs termed calDAG-GEFs. As the name suggests, these GEFs are regulated by calcium and/or DAG, via distinct binding domains (Stork, 2003). RapGTPase can be activated by the calDAG-GEFI (RasGRP2) and calDAG-GEFIII family members, and a recent study demonstrated that CXCL12-mediated B cell migration was dependent upon Rap activation, and in turn this activation was reliant on PLC activity (McLeod et al., 2002). Interestingly, calcium appeared not to participate in the activation of Rap in this study, correlating with CCR4-CEM cell chemotaxis being a calcium-independent migration event. The downstream effectors of Rap that may be involved in chemotaxis are not known. Effectors for Rap include PLC ϵ , p38, PI3K/PKB, Raf as well as possibly inhibiting PI3K/PKB, Raf and p38 (Stork, 2003). Recently, reports have indicated that Rap co-localises with F-actin and blocking its activation inhibited actin polymerisation (Caloca et al., 2004; McLeod et al., 2004). Also, CXCL12-stimulated Pyk2 tyrosine phosphorylation was attenuated with Rap inhibition (McLeod et al., 2004). Pyk2 is a non-receptor tyrosine kinase and member of the focal adhesion kinase (FAK) family. CCR4 ligation resulted in the PTX-sensitive tyrosine phosphorylation of Pyk2 and inhibition abrogates CXCL8-induced neutrophil chemotaxis, B cell and macrophage migration (Di, V et al., 2004; Guinamard et al., 2000; Okigaki et al., 2003).

Involvement of Alternative Pathways in CCR4-Mediated CEM Cell Migration

Work presented within this thesis additionally investigated the involvement of the p38-MAPK, JAK, and ERK-MAPK protein kinases in directed T cell migration in response to CCR4 ligands. The p38-MAPK has been shown to be required for CCL11/CCR3-mediated eosinophil chemotaxis, monocyte and neutrophil chemotaxis (Adachi et al., 2003; Ashida et

al., 2001b; Coxon et al., 2003). Although CCR4-ligation led to phosphorylation of p38, a selective inhibitor against this MAPK had no effect on CCR4-mediated cell migration. This effect compares favourably with other studies suggesting a lack of a role for p38 in cell migration (Fernandis et al., 2004; Ko et al., 2002).

Additionally, another MAPK family member ERK1/2 has been implicated in chemotactic events (Adachi et al., 2003; Brahmabhatt and Klemke, 2003; Coxon et al., 2003), and likewise with p38, inhibition of MEK1/2 (whose only known effector is ERK1/2) had no effect on CEM cell chemotaxis towards MDC, despite curtailing MDC-induced ERK phosphorylation events. The lack of effect of ERK inhibition on migratory events by the use of the MEK inhibitor PD98059 has been widely reported in the literature (Bardi et al., 2003; Cherla and Ganju, 2001; Fernandis et al., 2004; Fine et al., 2001; Ko et al., 2002; Vicente-Manzanares et al., 2002; Wang et al., 2002a). The JAKs have been extensively talked about previously in this discussion and, similar to effects on calcium mobilisation, their inhibition had no detrimental outcome on the chemotactic ability of CEM cells to migrate towards MDC, further discounting a role of these tyrosine kinase in the signalling and functional responses mediated by CCR4.

Finally, a very interesting response was obtained when Th2 cells were pretreated with an antibody against hMDC, resulting in an almost 100% increase in MDC-induced chemotaxis but with no effect upon TARC-stimulated chemotaxis. This spectacle is currently unexplainable and a number of experiments are required to be performed in order to try to understand this response. The antibody (anti-hMDC mAb, R & D Systems) is neutralising towards MDC activity with an ND₅₀ of 0.5 – 2 µg/ml (utilised at 10 µg/ml in assay). It is possible that the T cells themselves are producing MDC and that by preincubating them with anti-hMDC is mopping up secreted MDC and thus increasing the sensitivity of the receptor towards MDC when exposed to the gradient. If this is the case then it is interesting that mopping up the secreted MDC has no beneficial effect on TARC responses (T cells cannot produce TARC). One explanation is that the two chemokines are acting via different receptors, with TARC acting on an additional receptor for mediating chemotaxis of Th2 cells. This would help to explain why the *in vitro* generated Th2 cells chemotax to TARC but CEM cells do not (to any great extent), despite CEMs expressing CCR4 and

chemotaxing to MDC, as well as producing signalling responses to TARC. This could be easily confirmed/dismissed by the use of a neutralising antibody towards CCR4.

Signalling Pathways Leading to ERK and PKB Phosphorylation

As has previously been mentioned, the ERK-MAPK and PKB signalling cascades are two of the most widely studied signalling pathways, with both 'routes' and their downstream effectors implicated in a wide-range of cellular functions, including cell proliferation and survival. The ERK and PKB pathways are generally considered to be reasonably distinct from each other, although there have been reports that there is a level of cross-talk between the two. While these cascades are relatively well characterised in various systems there is not a lot known about how chemokine receptors regulate each pathway. Thus this section will discuss the results obtained for CCR4-mediated activation/phosphorylation of ERK and PKB and attempt to relate it to current knowledge for other chemokine receptors.

Characterisation of the CCR4 Activated ERK-MAPK Pathway

Not unexpectedly, CCR4 ligation led to the phosphorylation of ERK1/2 (both isoforms will subsequently be referred to as ERK) in a range of different cells, with broadly similar kinetics that is in contrast to those observed for PKB phosphorylation. As expected, incubation of cells with the selective MEK1/2 (referred to as MEK from this point forward) inhibitor PD98059 led to a complete abolishment of CCR4-induced ERK phosphorylation. This confirms the involvement of the Raf-MEK-ERK pathway in CCR4-mediated signalling. MEK is the only generally acknowledged substrate of Raf kinases, with MEK's only known substrate being that of ERK, whilst ERK has over 70 substrates, many of them nuclear transcription factors (O'Neill and Kolch, 2004). However, the molecular machinery that is responsible for activation of the Raf-MEK-ERK pathway, in response to chemokine stimulation, is lacking clarity and appears to almost certainly vary between the chemokine receptors and cell systems being studied.

Having established that ERK phosphorylation is sensitive to PTX treatment, *in vivo* evidence indicates that activation of ERK by G α i-coupled receptors is likely to employ the $\beta\gamma$ subunits of the heterotrimeric G-protein complexes (Koch et al., 1994; Luttrell et al., 1996). These $\beta\gamma$ subunits can then activate a range of molecules that lead to ERK activation. CXCL12-mediated stimulation of ERK in T lymphocytes has consistently been demonstrated to be a prolonged event (Ganju et al., 1998a; Kremer et al., 2003; Tilton et al., 2000) that relies upon the tyrosine kinase ZAP-70 and the scaffold/adaptor protein SLP-76 (SH2 domain-containing leukocyte protein of 76 kDa) (Kremer et al., 2003). However, rapid and transient ERK phosphorylation does not rely upon ZAP-70 and SLP-76, thus it is probable that these proteins are not responsible for CCR4-mediated ERK activation, which is transient. Alternative non-receptor tyrosine kinases that have been implicated in ERK phosphorylation, downstream of G α i $\beta\gamma$, are those of the Src family (Luttrell et al., 1996; Ma et al., 2000). Src (and other family members e.g. Lck and Lyn) have been implicated in the activation of the ERK pathway downstream of CXCR3 (Bonacchi et al., 2001) and demonstrated to be activated downstream of CXCR4 and CXCR2 (Inngjerdingen et al., 2002; Okabe et al., 2002; Ptasznik et al., 2002; Yamasaki et al., 2001). Moreover, Src tyrosine kinases have been shown to associate with Pyk2 and subsequent activation of the ERK pathway, among others, suggesting a potential role for CCR4-mediated Pyk2 phosphorylation in T lymphocytes (Avraham et al., 2000; Yamasaki et al., 2001). It is possible that Src activation by $\beta\gamma$ subunits does not involve a direct interaction and that perhaps PI3K γ serves as an intermediary (Hawes et al., 1996; Lopez-Illasaca et al., 1997). Indeed, there are a number of studies that report chemokine-mediated ERK1/2 phosphorylation is sensitive to treatment with PI3K inhibitors (Kansra et al., 2001; Knall et al., 1996; Sotsios et al., 1999). However, LY294002 and wortmannin treatments had no impact on MDC-stimulated ERK phosphorylation, therefore seemingly discounting a role for PI3K in this system.

In contrast, PLC inhibition and the attenuation of intracellular calcium mobilisation had small but significant ramifications on ERK phosphorylation downstream of ligated CCR4. It is possible that the mobilised calcium (generated by PLC activity) is required for Pyk2 activity and the subsequent formation of Shc-Grb2-Sos complexes that then activate Ras (a small GTPase) and the Raf-MEK-ERK pathway (that can incidentally be achieved via Src

and without the need for Pyk2) (Marinissen and Gutkind, 2001). Shc and Grb2 are SH2 domain-containing adaptor proteins and Src is able to phosphorylate Shc thus recruiting Grb2 and the RasGEF called Sos (son of sevenless). Alternatively, the rise in intracellular calcium could be activating Ras guanine-nucleotide releasing factor (RasGRF) or calDAG-GEFS with subsequent Ras stimulation. Although PKC isoforms have been established to activate the ERK pathway (in a Ras-independent manner) in various cell systems, including chemokine systems, the use of PKC inhibitors against the classical and novel PKC isoforms had no repercussions on CCR4-stimulated ERK phosphorylation (Ghosh et al., 2004; Kim et al., 2003; Woo et al., 2002). It is also possible that DAG, a product of PLC-mediated PI(4,5)P₂ hydrolysis, alone is activating RasGRP1 (Ras guanine-nucleotide releasing protein (calDAG-GEFII)) and/or RasGRP3. Both of these Ras activating proteins have been shown to be high-affinity DAG/phorbol-ester receptors and involved in the activation of the Ras-Raf-MEK-ERK pathway (Lorenzo et al., 2000; Lorenzo et al., 2001). Indeed, in thymocytes it has been demonstrated that a lack of RasGRP1 leads to the unresponsiveness of the cells in Ras signalling assays and proliferation experiments upon TCR ligation (Dower et al., 2000). Another possibility that relies on DAG is that of Rap-mediated ERK activation. This mechanism is Ras-independent with Rap activating Raf. However, the role of Rap varies between cell types and stimuli, with Rap capable of activating or inhibiting this route to ERK activation. Also, it is known that Rap is able to stimulate the activation of B-Raf, but B-Raf is not present generally in peripheral T cells, although its introduction leads to an ability for Rap to activate the ERK pathway (Dillon et al., 2003). There needs to be more investigation into the role of Rap in activating/inhibiting ERK in T cells, but current data would suggest that Rap is inhibitory downstream of the TCR (Appleman et al., 2001; Carey et al., 2000). Intriguingly, this may provide an explanation for the increase in basal levels of phosphorylated ERK upon treatment with the higher concentration of the PLC inhibitor U73122. It is possible that the basal inhibition of ERK phosphorylation is removed by inhibiting the basal levels of DAG thus inactivating Rap and leading to the unregulated activation of Raf and the consequent activation of MEK and ERK. It is also apparent that a chymotrypsin-like serine protease is involved in the homeostatic regulation of ERK as demonstrated by the increased levels of ERK phosphorylation observed with TPCK treatment in CEM cells. In contrast the trypsin-like serine protease inhibitor TLCK had no impact on ERK activity.

One of the first identified 70+ effectors of ERK was p90^{RSK} (RSK, MAPKAP-K1). This kinase is involved in the regulation of the cell cycle, gene transcription (including that of chemokines), and protein synthesis (Frodin and Gammeltoft, 1999; Zhang et al., 2002). MDC was able to stimulate the PTX- and PD98059-sensitive phosphorylation of p90^{RSK}, thereby identifying one such substrate of ERK that is activated upon CCR4 ligation. The only pharmacological inhibitor that produced a complete attenuation of ERK phosphorylation in response to CCR4 ligation was that of PD98059, as expected. Thus it is likely that there are a number of pathways upstream of Raf that contribute to the activation of the MEK-ERK cascade. Figure 4.52 is a schematic of the possible pathways, based upon the data presented within and from the current literature, which lead to ERK activation upon stimulation with MDC or TARC.

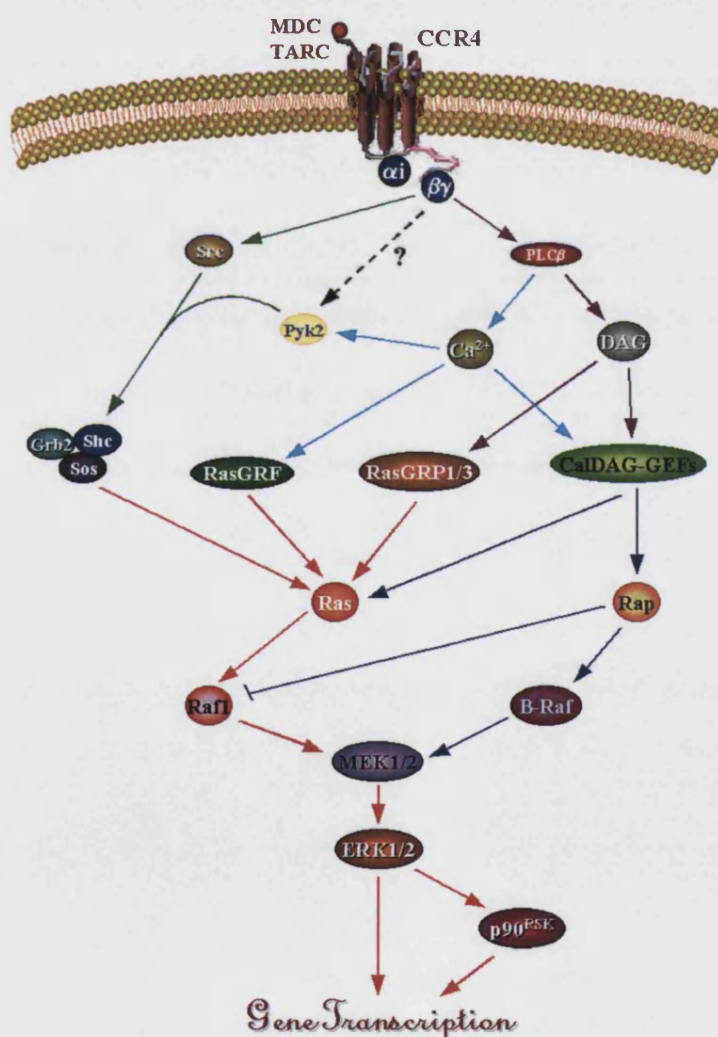


Figure 4.52. Schematic diagram of the possible pathways downstream of CCR4 that lead to ERK-MAPK activation. See text for more details.

Characterisation of the CCR4 Activated PI3K-PKB Pathway

The author refers the reader to 'Results I' and 'Discussion I' for an in depth analysis into the kinetics of PKB phosphorylation, and its dependency on PI3K activity, upon ligation of CCR4 with either MDC or TARC. This section will discuss the possible pathways that lead to PI3K activation and subsequent PKB phosphorylation.

As with all the other CCR4-mediated signalling cascades investigated within this thesis, CCR4-induced PKB phosphorylation is sensitive to PTX treatment indicating the requirement for CCR4 to couple to the $G\alpha_i$ protein. There are likely to be a number of routes that lead to the activation of PI3K and subsequent activation of PKB. The most straight forward of these would involve the direct activation of PI3K γ by the $\beta\gamma$ subunits. PI3K γ is the only isoform that can be activated in this way by GPCRs and it was earlier shown that PI3K γ is indeed activated by MDC in CEM cells. This activation of PI3K γ will lead to an increase in PI(3,4)P₂ and PI(3,4,5)P₃ lipids, recruitment of PDK-1 and subsequent recruitment/activation of PKB and its downstream effectors (Brock et al., 2003; Wymann et al., 2003a). The activation of the class IA PI3K isoforms cannot occur directly via the $G\beta\gamma$ proteins but instead can occur via a classical pathway involving the recruitment and activation of a Src tyrosine kinase (by either $G\alpha_i$ or $\beta\gamma$) that then tyrosine phosphorylates the adaptor protein Shc with subsequent recruitment of Grb2 and the RasGEF Sos (Ma et al., 2000; Stephens et al., 1993). This leads to the activation of Ras that can then activate, via its association with the catalytic domain, the class IA PI3K isoforms (Kodaki et al., 1994; Rodriguez-Viciana et al., 1994; Vanhaesebroeck et al., 1997a). Ras and the adaptors play a role in recruiting PI3K to the plasma membrane where it can phosphorylate its main substrate PI(4,5)P₂. However, the exact mechanisms surrounding class IA PI3K recruitment downstream of chemokine receptors is ill-defined (Cantley, 2002; Ward and Cantrell, 2001). Furthermore, it has been established that Ras can additionally activate PI3K γ (Suire et al., 2002). This finding may be crucial as it has been determined within this thesis that phosphorylation of PKB is dependent upon PLC activity, suggesting that PI3K γ does not contribute to PKB activation if it is solely activated in a direct manner by the $G\beta\gamma$ subunits. In contrast however to ERK phosphorylation, calcium mobilisation was surplus to requirements with regard to CCR4-mediated PKB

phosphorylation. Therefore, not unexpectedly, the selective classical PKC inhibitor had no effect on levels of phosphorylated PKB, but the broad-spectrum PKC inhibitor RO-32-0432 did have a slight negative impact. This indicates that a novel PKC isoform is involved in PKB activation and the options would appear to be restricted to PKC θ , PKC η or PKC ϵ , as the PKC δ inhibitor rottlerin had no impact. The role of PKC isoforms in the PI3K-PKB cascade remains ambiguous but there are studies that examine this relationship. Classical and novel PKC isoforms have been described as influencing in a positive manner the phosphorylation of PKB (Bauer et al., 2003; Glikli et al., 2002; Kim et al., 2004; Matsumoto et al., 2001). Although there is a requirement for PLC (and likely its products) it cannot be ruled out that a DAG-dependent pathway separate from novel PKC isoform activity is involved. Since RO-32-0432 displays activity against the atypical PKC λ (no data available for PKC ζ), it is possible that an atypical isoform is involved in modulating the activity of PKB. Indeed, atypical isoforms have been implicated as being negative regulators of PKB activity (Doornbos et al., 1999; Mao et al., 2000) but, importantly for this study, have also been described as having a positive role (Li et al., 2004a; McConkey et al., 2004). It is not clear how PKC is mediating these effects. One explanation is that PKC is constitutively bound to PKB and induces its recruitment to the membrane as has recently been shown to occur for PKC θ upon activation of CD3 in T cells, whilst other PKC isoforms are involved in the transactivation of PKB (Bauer et al., 2003; Konishi et al., 1994). It is also possible that PKC is only having an impact at the level of Ser 473 phosphorylation and is not affecting Thr 308 phosphorylation (Kroner et al., 2000), although this is in contrast to the data presented by Kim et al. (Kim et al., 2004). PDK-1 is known to be involved in the activation (by phosphorylation of the activation loop) of PKC isoforms, with certain isoforms observed to directly interact with PDK-1, in various circumstances and this mechanism would fit in nicely with the observation that PKB phosphorylation is fully dependent on PI3K activity (Cenni et al., 2002; Chou et al., 1998; Dutil et al., 1998). RO-32-0432 did not completely attenuate PKB phosphorylation at the Ser 473 residue and maximal effects were seen at sub-maximal utilised concentrations. This would suggest that PKC is not required for complete activation of PKB. Therefore, an alternative explanation for the role of DAG is required as PLC activity is essential for PKB phosphorylation. It is possible that DAG is activating a RasGEF, such as the CalDAG-GEFs or RasGRPs, with subsequent activation of Ras and recruitment of PI3K. These pathways have been

discussed earlier in this discussion and will not be covered again. An absolute requirement of PLC activity for PKB activation has been demonstrated in two other systems, with one of these studies determining calcium was the essential product and the other established a role for PKC (Amin et al., 2003; Tang et al., 2002).

Surprisingly, both TLCK and TPCK abrogated MDC-induced PKB phosphorylation. A paper published in 2001 detailed the ability of TPCK to disrupt PDK-1 signalling by preventing PDK-1 from phosphorylating its regulatory sites in PKB (phosphorylation at Ser 473 and Thr 308 was inhibited indicating PDK-2 inhibition as well) (Ballif et al., 2001). It is not known about the sensitivity of PDK to TLCK. It is generally considered that I κ B kinase is a downstream target of PKB. However, another intriguing paper has suggested that NF- κ B is upstream of PKB and inhibition of NF- κ B (with TPCK and SN-50) led to the inhibition of PKB whilst PI3K inhibition had no effect upon NF- κ B activity (Meng et al., 2002). Additionally, this report demonstrated that over expression of p65 (of NF- κ B) leads to higher PKB phosphorylation (24 hr) and an increase in PKB mRNA (at 12 hr). Phosphorylation of PKB in this study was slow (TNF = 15 min; LPS = 60 min) and prolonged and would suggest that although NF- κ B may well be able to activate PKB in certain situations it is unlikely to be responsible for MDC- and TARC-mediated PKB phosphorylation, which is a very rapid event. As such, it is more likely that TPCK and TLCK are mediating their effects via the disruption of PDK-1's (and 2's) ability to phosphorylate PKB.

An established substrate of PKB is GSK-3 and, unusually, this leads to the inactivation of GSK-3, with a subsequent increase in the activity of glycogen synthase (Cohen and Frame, 2001). MDC-stimulated phosphorylation of GSK-3 α/β was indeed sensitive to LY294002 but this was not absolute. However, treatment of cells with the PKC inhibitor RO-32-0432 prior to MDC stimulation did result in the abolition of GSK-3 α/β phosphorylation. This is not a surprise with PKC an established activator of GSK-3 (Christian et al., 2002; Fang et al., 2002; Tsujio et al., 2000). Figure 4.53 is a schematic of the pathways that possibly operate upon CCR4-ligation and that lead to activation of PKB.

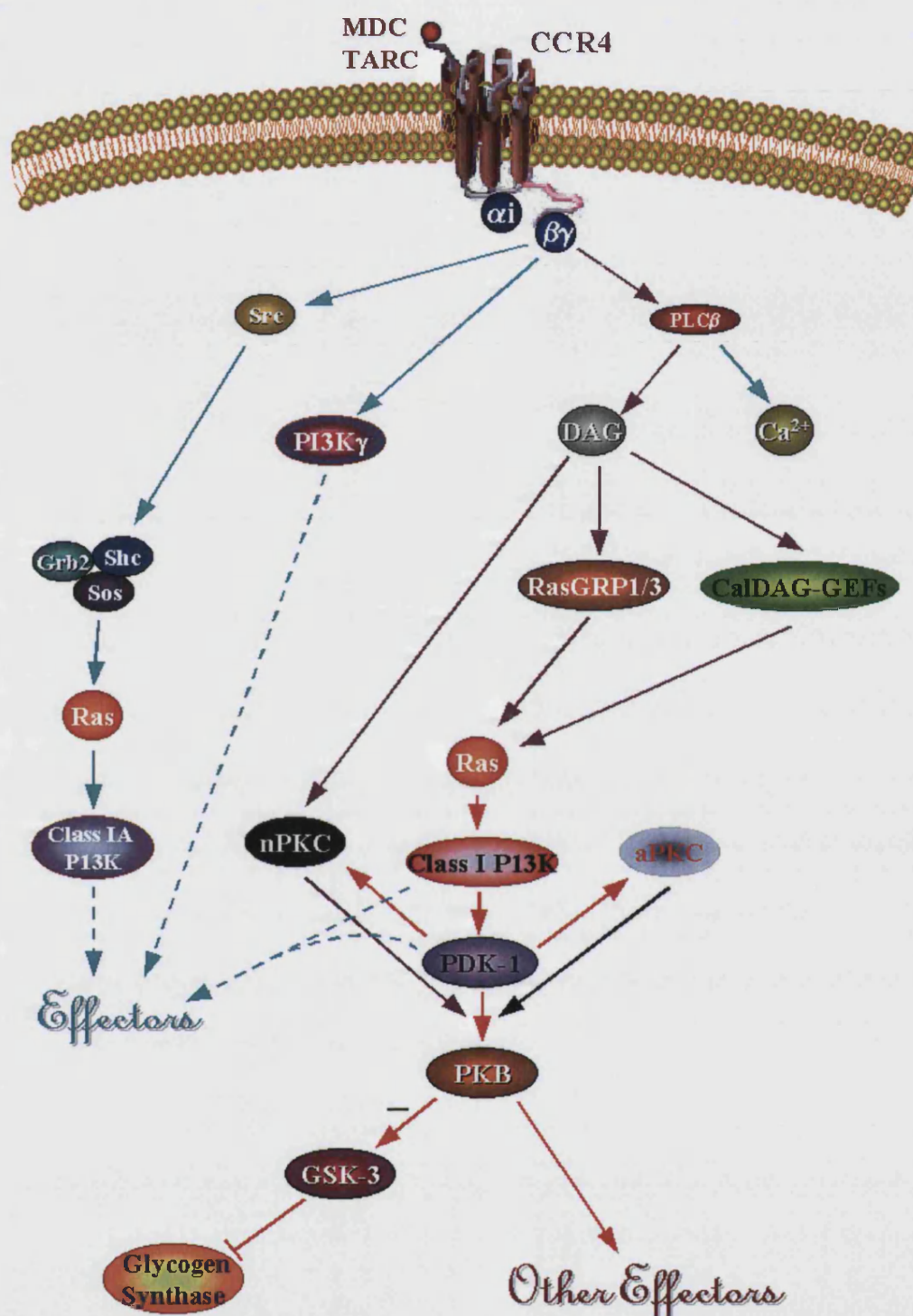


Figure 4.53. Schematic diagram of the potential CCR4-mediated signalling cascade that leads to PKB phosphorylation. ‘Other effectors’ include I κ B kinase, eNOS, mTOR, Bad, FKHR, caspase 9 (last 3 are inhibited). ‘Effectors’ include p70S6K, Rac/RhoGEFs, Tec kinases (e.g. Btk, Itk, Tec). See text for more details of the cascade. Dotted aqua lines indicate pathways that have not been supported by data presented within this thesis with respect to phosphorylation of PKB but may occur to activate other PI3K-dependent pathways.

Possible Role of Arno and Identity of UPE

Identifying a Role for Arno

Arno (ARF nucleotide binding site opener) is a GEF for the GTPases ARF1 and ARF6. These ARF GTPases are best known for their role in vesicle transport, with ARF1 localising primarily to the Golgi complex where it initiates carrier vesicle formation by nucleating the assembly of coat protein complexes (Donaldson and Klausner, 1994). Contrastingly, ARF6 has been localised to the plasma membrane/endosomal system, where it regulates the recycling of membrane proteins (Chavrier and Goud, 1999). It has been demonstrated that ARF6 also regulates the availability of β -arrestin to GPCRs with receptor activation leading to receptor desensitisation by β -arrestin (which has been liberated from activated ARF6) sterically hindering the ability of the receptor to activate its cognate G protein (Hunzicker-Dunn et al., 2002). Additionally, ARF6 has been shown to be required for membrane ruffling and lamellipodia formation, with ARF6 colocalising with Rac1 and required for Rac1 recycling to the plasma membrane (Radhakrishna et al., 1999; Song et al., 1998; Van Aelst and D'Souza-Schorey, 1997). Arno is a 47 kDa protein that possesses a C-terminal PH domain. The selectivity of this PH domain appears to be broad as it has been demonstrated that it binds PI(4,5)P₂ and PI(3,4,5)P₃ (Chardin et al., 1996; Paris et al., 1997; Venkateswarlu et al., 1998). This perhaps suggests that although PI3K activity is not required for T cell chemotaxis in response to MDC or TARC, it does not rule out a role for Arno and ARF6. Arno has been shown to colocalise with ARF6 in actin-rich lamellipodia at the cell periphery, but only upon treatment with phorbol ester (Frank et al., 1998). Arno has been shown to stimulate epithelial cell migration through stimulation of Rac1 and PLD, however in the few PLD assays performed MDC was unable to initiate a detectable increase in PLD activity in CCR4 transfected CHO cells (data not presented) (Santy and Casanova, 2001). Although this would suggest that Arno is not working through the PLD pathway it is necessary to perform a more in depth analysis into PLD activation in T cells before any substantial conclusions can be drawn. The review by Turner & Brown goes into more detail about the possible role of Arno and PLD in cell motility (Turner and Brown, 2001). It is impossible to define the exact role Arno and its substrates ARF1/6 are performing in CCR4-mediated signal transduction, but it is possible that Arno/ARF could

be involved in one or more of the mechanisms mentioned above and only future studies would help to clarify this.

Possible Candidates for the Identity of UPE

The kinetics for UPE phosphorylation are relatively slow at 5 – 10 minutes. This would suggest that UPE is reasonably far down the CCR4-initiated signalling cascade in T cells. Additionally, it is a very large molecule at about 200 kDa helping to considerably cut down on the number of potential candidates. Since the antibody used was against FKHR (FOXO1) phosphorylated at Ser 256 then potential candidates will include members of the FOXO family of transcription factors: AFX (FOXO4) and FKHR-L1 (FOXO3a). These transcription factors all possess the conserved serine residue at the N-terminal end of the DNA-binding domain. However, AFX and FKHR-L1 are only 65 and 97 kDa, respectively, ruling out these factors as possible candidates. The serine residue at 256 lies within a consensus sequence for phosphorylation by PKB, therefore possible candidates for UPE should include this consensus region, such as caspase 9 (47 kDa), Bad (23 kDa), IKK α (not clear (85 kDa)), GSK-3 α/β (54/46 kDa), PDE3B (124 kDa), mTOR (289 kDa), IRS-1 (175 kDa), Raf (74 kDa), eNOS (140 kDa) and BRCA1 (220 kDa). Of these proteins, IRS-1, mTOR and BRCA1 are potential candidates with regard to their size. However, IRS-1 (insulin receptor substrate-1) is a little too small and unlikely to be activated downstream of a chemokine receptor in T cells; mTOR (molecular target of rapamycin) is a distinct possibility but an anti-phospho mTOR Ab has been used and there was considerable basal levels of phosphorylated protein (data not shown); BRCA1 (breast-cancer-susceptibility-gene-1 product) is a possibility. BRCA1 is a nuclear phosphoprotein, thought to be a tumour suppressor, expressed in T lymphocytes and is encoded by the *BRCA-1* gene and activated by PKB (Green and Schuler, 2000; Vanhaesebroeck and Alessi, 2000). Another possibility is tuberlin (200 kDa, also known as TSC2), a product of the tumour suppressor gene *TSC2*, involved in regulating cell proliferation and tumour development. Tuberlin is directly phosphorylated by PKB and is believed to be inhibited by this event, and thereby possibly stimulating the mTOR pathway (Li et al., 2004b). This is perhaps a more viable candidate for UPE as mTOR has been implicated in chemokine signalling (Hwang et al., 2003). However, it is perhaps more likely that it is none of the above-mentioned proteins and only sufficient analysis will determine its true identity.

Conclusions II

The second section of this thesis has concentrated primarily on the pathways that may be required for CCR4-mediated T cell chemotaxis. It has been demonstrated that CCR4 signalling and chemotaxis requires the coupling of the receptor to the G α i protein and that the Janus kinases appear to have no part to play. MDC and TARC are able to mobilise calcium from intracellular IP3R-sensitive stores and that other mechanisms of calcium release appear to have little or no contribution towards the total cytoplasmic levels. PLC is a pivotal enzyme that is required for calcium, PKB signalling and chemotaxis, however, its involvement in the ERK-MAPK pathway is ambiguous. Intracellular calcium mobilisation is redundant with respect to a cell's ability to directionally migrate towards MDC. Moreover, a calcium flux is not required for the phosphorylation of PKB although it does contribute to a certain extent in the activation of ERK. The other product of PI(4,5)P₂ hydrolysis by PLC, DAG, appears to be a pivotal player in chemotaxis and PKB signalling and likely to involve effectors that are not only members of the PKC family. However, although CCR4-ligation leads to activation of the ERK-MAPK pathway (and PKB pathway, as discussed previously) it is not involved in events required for cell migration. Instead, it has been established that the Rho-ROCK-MLC pathway is at least one cascade that is indispensable for CCR4 directed T cell migration. In addition, there have been a number of other pathways discussed that may have crucial roles to play in the chemotaxis of T cells.

Key Future Experiments II

1. Perform a more detailed analysis of the role of PKC and/or DAG in the signalling and chemotactic responses. This will involve using DAG and OAG (a DAG that activates only calcium-dependent PKCs (1-Oleoyl-2-acetyl-*sn*-glycerol)) to stimulate or downregulate (through prolonged exposure) PKC isoforms. Additionally, look at cell localisation of the PKCs (this was investigated briefly but could observe no changes) and their phosphorylation state in Th2 cells (phosphorylated basal levels are too high in CEMs). Also, to utilise siRNA technology and/or peptide inhibitors to selectively inhibit individual PKC isoforms. Using DAG/OAG in presence of PKC inhibition will help to determine the presence of non-PKC-mediated pathways, in addition to targeting specific DAG effectors.
2. Investigate more thoroughly the role of NF- κ B in the chemotactic response and, if any, the genes that are transcribed.
3. Characterise the effectors of the calcium pathway e.g. calmodulin, NFAT, etc.
4. Investigate the Rho-ROCK-MLC pathway more thoroughly and establish the role of the 'other' GTPases cdc42 and Rac in actin polymerisation and cell migration utilising techniques, including confocal microscopy, to determine co-associations.
5. Characterise pathways other than PKB and ERK that are activated upon CCR4 ligation. Determine the range of effectors activated by the ERK and PKB pathways, as well as their transcription factors. Attempt to relate this to their role in CCR4 function.
6. Determine role of DOCK2 in CCR4-mediated cell migration of T cells.
7. Establish the identity of UPE through protein isolation/purification and sequencing.
8. Ascertain the role of Arno/ARF in CCR4-mediated responses and whether it is required for cell motility.
9. Verify whether MDC and TARC stimulate identical signalling pathways, and if CCR4-mediated T cell chemotaxis utilises the same signalling cascades as other CCR4-expressing cells e.g. monocytes, DCs, thymocytes.

5

OVERALL DISCUSSION & CONCLUSIONS

5. OVERALL DISCUSSION & CONCLUSIONS

5.1 Potential Roles of Calcium, ERK and PKB in CCR4 Functions

It has been established through the course of this thesis that each of the calcium-, ERK- and PKB-associated signalling cascades is dispensable for CCR4-mediated directed T cell migration. However, it is unlikely that the activation of these pathways are a redundant feature of chemokine activity and more plausible that, despite their name, chemokines have a much broader repertoire with regard to their functional role than simply an ability to elicit a cell migratory response. The following few sub-sections will very briefly discuss the potential role these three pathways may have in a cell's actions.

Calcium

Calcium is an incredibly versatile intracellular signal that regulates a plethora of different cellular functions. Mobilised calcium exerts its effects via EF-hand proteins (see appendix 9) that bind calcium with high affinity to helix-loop-helix motifs that are repeated from two to twelve times. A ubiquitous and abundant EF-hand protein is calmodulin (CaM) that interacts with and regulates various proteins including calmodulin-dependent kinases (CaMKs), calcineurin, NFAT and AP-1. When intracellular calcium levels are low CaM assumes a closed-conformation that prevent it from binding to protein kinases. An increase in intracellular calcium leads to CaM binding 4 calcium ions and subsequent conformational change allowing CaM to relieve the autoinhibition of its target kinases. One such target of CaM is calcineurin (protein phosphatase 2B), a serine/threonine phosphatase that binds directly to the transcription factor NFAT (nuclear factor of activated T cells) leading to its dephosphorylation allowing it to translocate from the cytosol to the nucleus (Hogan et al., 2003; Ikura et al., 2002). Additionally, CaMKs can activate the transcription factor CREB (cAMP response element binding protein) and SRF (serum response factor) (fig. 5.1). It has been shown that NFAT regulates the expression of various cytokines, such as IL-2, IL-4, IL-12 and TNF α (Luo et al., 1996; Rao et al., 1997). Calcineurin has also been demonstrated to be involved in the negative regulation of

CXCR4 expression in T cells (Cristillo and Bierer, 2003), and NFAT is involved in the regulation of chemokine (CCL4, CCL2) production by GPCR (fMLP) activation (and required the synergistic interaction of the ERK pathway) (Ali et al., 2000). Currently there is sparse data regarding the role of chemokine-activated NFAT (if it is even activated) but it is possible that chemokine stimulation may lead to changes in expression of various chemokine receptors and chemokine expression profiles i.e. T cell differentiation, and involved in T cell proliferation via increased secretion of cytokines e.g. IL-2. Any effect calcium is having on transcription is in addition to the effects it has in mediating classical PKC, Pyk2 and other kinase activation. Calcium is additionally thought to have a substantial role in cell adhesion and it is possible that in transendothelial migration studies, where adhesion will be a part, calcium may be required for chemokine-mediated migration (Hofman et al., 1999; Rouleau et al., 2003).

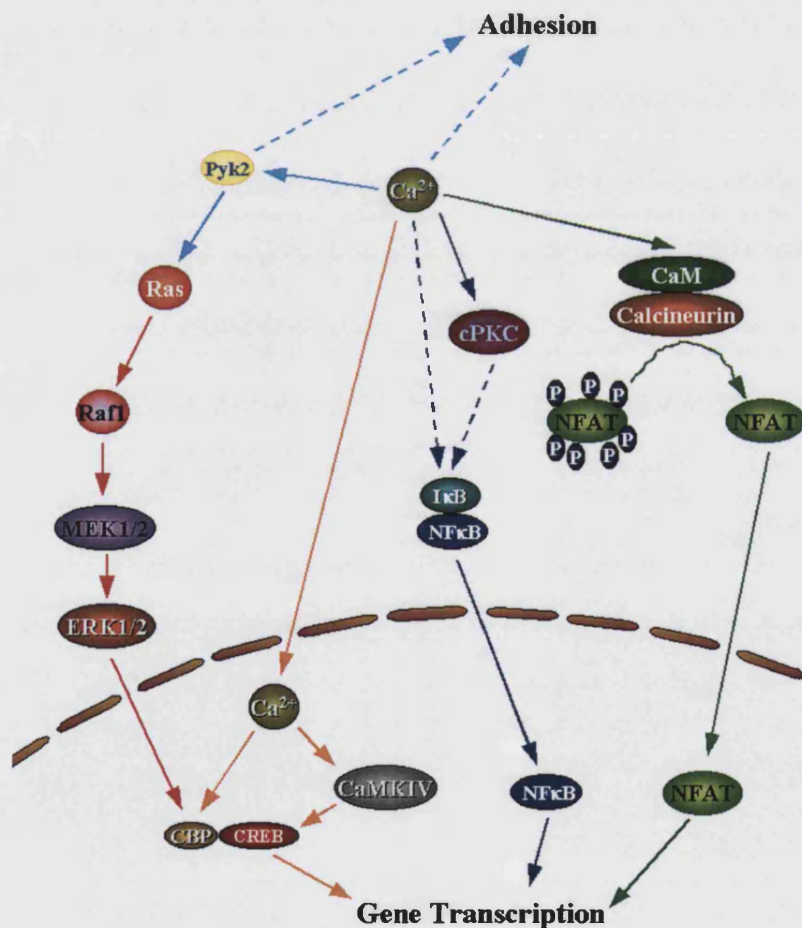


Figure 5.1. A selection of possible calcium-influenced pathways activated by CCR4 in T cells. See text for more details. Abbreviations (not explained in text): cPKC, classical PKC isoforms; CBP, CREB-binding protein; P = phosphorylated serines.

ERK

The ERK-MAPK cascade is a major player in regulating gene expression in eukaryotic cells, with ERK able to activate a plethora of substrate proteins that include MSK1/2 (mitogen- and stress-activated kinase 1/2), p90^{RSK}, MNK1/2 (MAP kinase interacting kinase 1/2), SRF, AP-1, CREB, Histone H3, Ets, Elk-1, STAT1-3 and c-myc/n-myc (Waas et al., 2004). Transcription factors such as Elk-1, c-myc and CREB are generally thought to be involved in cell proliferation and differentiation and various chemokines have been demonstrated to activate these transcription factors (e.g. Elk-1, CREB, AP-1, c-myc) (Kim et al., 2003; Lentzsch et al., 2003; Majka et al., 2000b; Rincon, 2001; Schaeffer and Weber, 1999; Suzuki et al., 2001; Yang et al., 2001; Zhang et al., 2002). Thus, chemokines may serve to amplify the immune response and as a consequence, in certain cases, prolong the inflammatory reaction via the activation of the ERK-MAPK pathway. Additionally, ERK activation of p90^{RSK} can lead to the inhibition of the pro-apoptotic protein Bad, thereby promoting cell survival.

PKB

Following its activation, PKB transduces signals that regulate multiple biological processes via an abundance of effectors (see 'Introduction' for a sample of these). PKB is widely considered to be involved in processes that include apoptosis, gene expression and cellular proliferation. Indeed, a number of chemokines have been shown to inhibit apoptosis in a PI3K-PKB-dependent manner, including CCL25 (CCR9), CXCL12 and CCL19/21 (CCR7) (Hideshima et al., 2002; Sanchez-Sanchez et al., 2004; Youn et al., 2002). Furthermore, CXCL12 has been determined to phosphorylate (thereby inhibiting) Bad in a PI3K-dependent manner in T cells (Hideshima et al., 2002; Suzuki et al., 2001). The anti-apoptotic effects of CCL19/21 were established to be via the activation of NF- κ B in mature DCs, probably via a PI3K regulated pathway (Sanchez-Sanchez et al., 2004). Additionally, a number of chemokines can promote proliferation in a PI3K-dependent manner, including CXCL16 (Chandrasekar et al., 2003a). Finally, CX3CL1 is able to induce its own expression via the PI3K-PKB-NF- κ B signalling cascade (Chandrasekar et al., 2003b). It is conceivable that CCR4 ligation is likely to deliver a pro-survival signal to the cell and

generate a proliferative response. CCR4-mediated PKB activation can also potentially have a role to play in other processes, such as vesicular trafficking (Chan et al., 1999).

5.2 Potential Signalling Pathways Required for CCR4-Mediated T Cell Chemotaxis

As has been discussed earlier, there is likely to be more than one signalling cascade required for cell motility in response to a chemotactic gradient. The motile cell performs a crawling movement with pseudopodia, detachment and contraction closely co-ordinated with one another to provide a smooth and efficient process. This thesis has found at least one pathway that is essential, namely the Rho-ROCK pathway. Other pathways that have been established to be required for a cell's ability to move in response to various stimuli (not necessarily to chemokines) include the cdc42 and Rac pathways, which are purported to control filopodia and lamellipodia formation, respectively, via actin polymerisation/depolymerisation processes (Haddad et al., 2001). These cascades are complex and beyond the scope of this thesis. In brief, the cdc42 GTPase binds to the Wiskott-Aldrich syndrome protein (WASP), which recruits ARP2/3 (actin-related protein 2/3) complex and initiates the barbed-end growth of actin filaments by enhancing nucleation. Similarly, Rac binds the WASP family protein WAVE, which also recruits ARP2/3 to form new barbed ends. Rac is additionally able to recruit the serine/threonine kinase PAK (p21-activated kinase) that co-localises with F-actin in membrane ruffles and to lamellipodia, where it helps mediate actin assembly. PAK can additionally phosphorylate LIM kinase (LIMK) that then phosphorylates and inactivates cofilin, thereby inhibiting cofilin's actin depolymerising and severing activity. The activity of cofilin is required though to eventually block lamellipod extension and this maybe via a PLC-dependent phosphatase (and may also require PKC activity) that relieves the inhibition of cofilin (Matsui et al., 2001; Zhan et al., 2003). Blocking actin depolymerisation will inhibit polarised (but not random) lamellipodia extension demonstrating the importance of concomitant depolymerising activity in the lamellipod (Cramer, 1999; Zebda et al., 2000). CXCL12 has been reported to activate LIMK and subsequent phosphorylation of cofilin in T cells and is required for chemotaxis (Nishita et al., 2002). Finally, the 140 kDa formin

mDia can regulate actin polymerisation through its interaction with the actin-polymerising protein profilin. Profilin has been shown to be involved in T cell migration through regulation of the F-actin/G-actin ratio, thereby modulating the availability of actin for other effectors (e.g. such as those involved in lamellipodia formation) (Vicente-Manzanares et al., 2003). There are a number of excellent reviews that cover the roles of cdc42/rac signalling in migration and the reader is encouraged to read these in order to obtain an overall understanding of the complexities of the pathways that are likely to be involved in T cell chemotaxis (Burridge and Wennerberg, 2004; Chung et al., 2001; Matozaki et al., 2000; Raftopoulou and Hall, 2004; Samstag et al., 2003; Sanchez-Madrid and del Pozo, 1999; Small et al., 2002). Figure 5.2 incorporates these cascades, along with the essential pathways reported within this thesis, in to a schematic diagram of potential/required CCR4 signal transduction in T cell chemotaxis. The proposed mechanism of filopodia/lamellipodia formation by actin polymerisation is presented in figure 5.3.

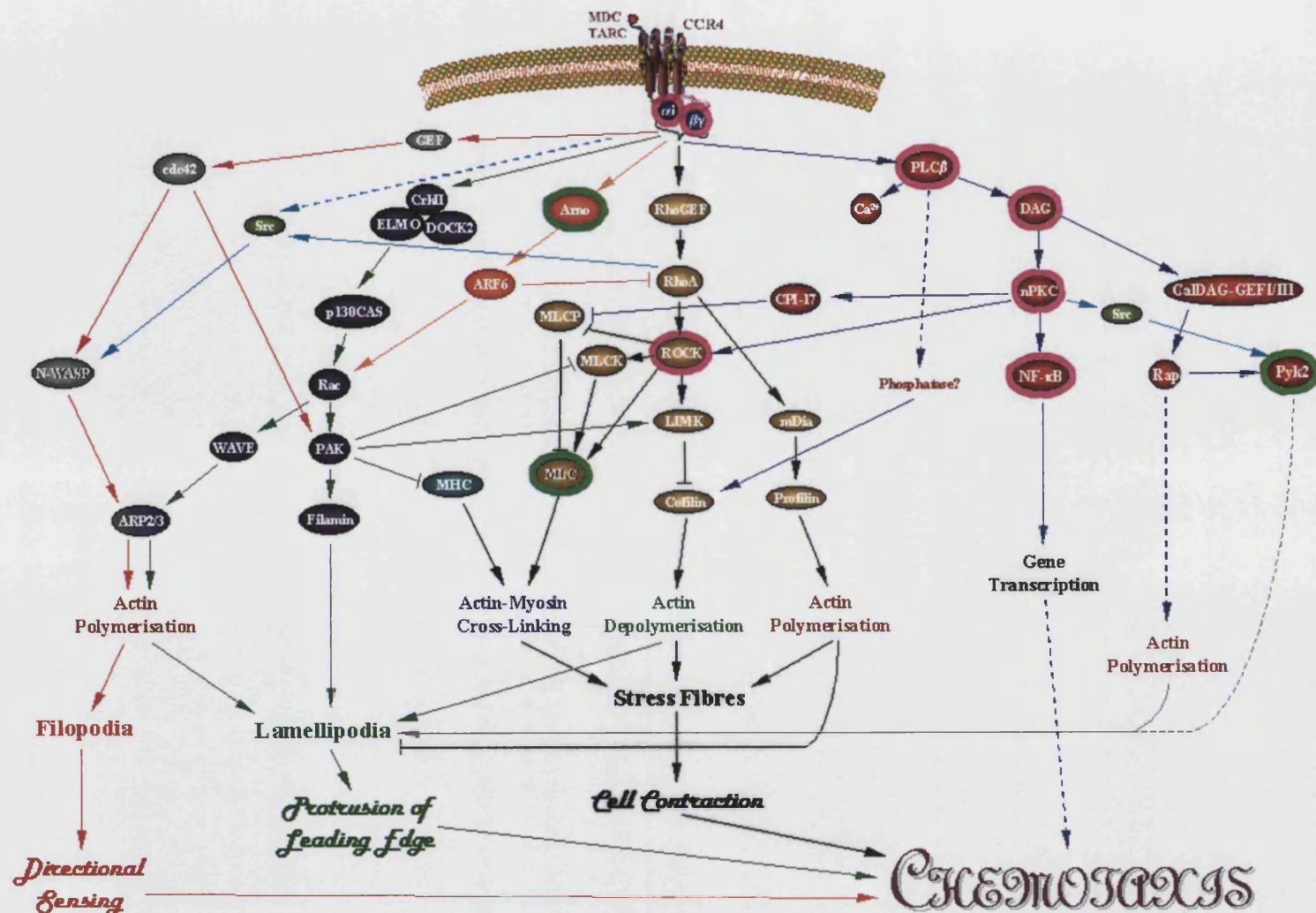


Figure 5.2. Potential pathways involved in CCR4-mediated 1 cell chemotaxis. Activation of molecules highlighted in pink is essential for CCR4-mediated 1 cell chemotaxis (from results presented within thesis), whilst molecules highlighted in green have been shown to be downstream of ligated CCR4. Dotted lines represent possible indirect routes. This is by no means a comprehensive model of the signalling cascades and the level of cross-talk in T cell chemotaxis, but does highlight some of the major routes and potential interactions. *Abbreviations (not defined within text):* CAS, Crk-associated substrate; ELMO, engulfment and cell motility (CED-12); LIMK, Lin-11 Isl-1 Mec-3 kinase; MHC, myosin heavy chain; WAVE, WASP family verprolin-homologous protein.

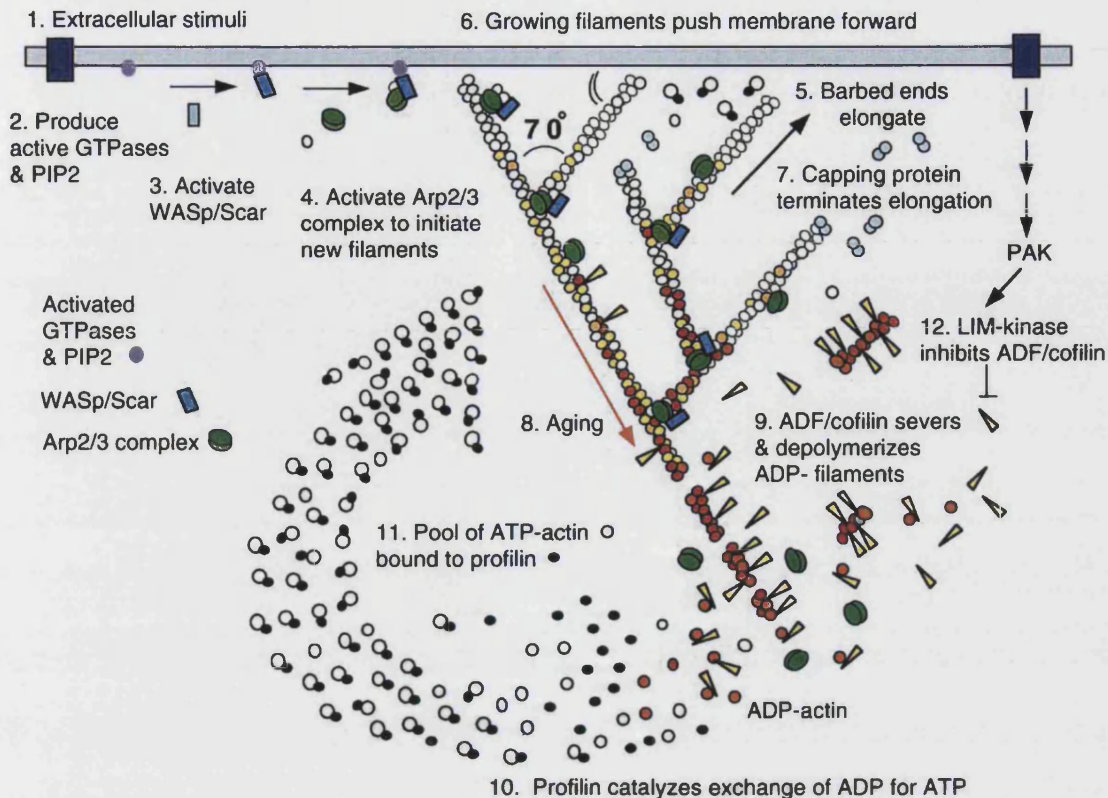


Figure 5.3. Dendritic nucleation/array (process of forming actin filament branches) treadmilling model for protrusion of the leading edge. (1) Extracellular signals activate receptors. (2) The associated signal transduction pathways produce active Rho-family GTPases and PIP2 that (3) activate WASp/Scar proteins. (4) WASp/Scar proteins bring together Arp2/3 complex and an actin monomer on the side of a pre-existing filament to form a branch. (5) Rapid growth at the barbed end of the new branch (6) pushes the membrane forward. (7) Capping protein terminates growth within a second or two. (8) Filaments age by hydrolysis of ATP bound to each actin subunit (white subunits turn yellow) followed by dissociation of the γ -phosphate (subunits turn red). (9) ADF/cofilin promotes phosphate dissociation, severs ADP-actin filaments and promotes dissociation of ADP-actin from filament ends. (10) Profilin catalyses the exchange of ADP for ATP (turning the subunits white), returning subunits to (11) the pool of ATP-actin bound to profilin, ready to elongate barbed ends as they become available. (12) Rho-family GTPases also activate PAK and LIM kinase, which phosphorylates ADF/cofilin. This tends to slow down the turnover of the filaments (Pollard and Borisy, 2003).

5.3 Overall Conclusion

As has been mentioned before, the pathways stimulated upon CCR4 ligation have not been examined, which provided an exciting window of opportunity in which to investigate. The results presented within this thesis have established, for the first time, a number of CCR4-induced signalling cascades, including that of the PI3K-PKB, ERK-MAPK and Rho-ROCK-MLC pathways, in T lymphocytes (or for any cell). Furthermore, a reasonably comprehensive examination of CCR4-mediated intracellular calcium mobilisation has been performed, and established that a calcium flux is redundant in the chemotactic response to MDC. This study also provides, for the first time, strong evidence for the lack of participation of PI3K in certain chemokine-mediated T cell migratory events (in this case CCR4). In comparison to results published for other receptors, it is clear that chemokines generally activate similar cascades with minor variations. However, the relevance of these cascades towards a cell's migratory response appears to vary quite considerably, with the most notable differences being the requirement (or lack of) PI3K and DOCK2 activity. It is very likely that as the chemokine signalling field develops more and more, variations will be observed in the signalling cascades that are activated/inhibited, along with disparities in those pathways required for cell migration. It will be intriguing to learn whether CCR4 signalling is similar in lymphocytes as in myeloid cells (e.g. monocytes) and whether there is a similar lack of necessity for lipid PI3K activity. It will also be interesting to elucidate whether PI3K is participating in chemotaxis independently of its lipid kinase activity. The work presented within has utilised, to a great extent, a variety of pharmacological inhibitors. It needs to be stressed that the majority of inhibitors are limited by their specificity, but if used at rational concentrations they can provide a good indication of a target molecule's requirement in a signalling pathway/response. Throughout this thesis inhibitors have been used at concentrations deemed to be selective for their target, as reported in the literature.

It is clear that, although this thesis has provided a greater understanding in to the pathways that regulate cell migration, there are still a vast number of unanswered questions remaining concerning the cascades involved in chemotaxis and the role of various pathways in non-chemotactic events. However, this thesis has hopefully provided some strong foundations for which future work can be built on.

6

APPENDICES

6. APPENDICES

6.1 Appendix 1: Recipes

Recipes for SDS-PAGE and Western Blotting

4 x Resolving Gel Buffer

	g/500 ml	
1.5 M Tris	90.86	pH 8.8
0.4% SDS	2.00	

4 x Stacking Gel Buffer

	g/500 ml	
0.5 M Tris	30.29	pH 6.8
0.4% SDS	2.00	

Formulae for SDS-PAGE Gel Solutions made with resolving and stacking gel buffers containing 0.4% SDS (SDS at a final concentration of 0.1% for all gels):

	Resolving Gel (20 ml)						Stacking Gel (12 ml)	
	5%	7.5%	10%	12%	14%	15%		5%
MQH₂O	11.51 ml	9.84 ml	8.17 ml	6.84 ml	5.51 ml	4.84 ml		6.85 ml
Resolving gel buffer (pH 8.8)	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml	5.0 ml	Stacking gel buffer (pH 6.8)	3.0 ml
Bis-Acryl (30%)	3.33 ml	5.0 ml	6.67 ml	8.00 ml	9.33 ml	10.00 ml		2.0 ml
10% APS	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl		150 µl
TEMED	15 µl	15 µl	15 µl	15 µl	15 µl	15 µl		15 µl

10% Ammonium Persulphate (APS)

1 g APS in 10 ml MQH₂O

Reservoir Buffer

	g/5L
25 mM Tris	15.15
192 mM glycine	72.00
0.1% SDS	5.00

Semi-Dry Transfer Buffer

	g/L
48 mM Tris	5.81
39 mM glycine	2.93
SDS	0.375
Methanol	200 ml

Transfer conditions: 0.8 mA/cm², i.e. 163.2 mA for 4 x (8.5 x 6.0) cm gels.

10 x Washing/Blocking Buffer

	g/L	
10 mM Tris	12.11	pH 7.5
100 mM NaCl	58.44	
0.1% Tween-20 (TBST only, omit for TBS)	10.0 ml	

For blocking add appropriate reagent e.g. 5% (w/v) non-fat milk powder, or 1% (w/v) BSA (see Appendix 5).

10% Sodium Azide

1 g NaN₃ in 10 mls MQH₂O (remember: do not use a metal spatula to weigh out NaN₃)

Stripping Solution

	100 ml
100 mM 2-mercaptoethanol (stock: 14.3 M)	699 μ l
2% SDS	2 g
62.5 mM Tris-HCl (stock: 200 mM, pH 6.8 – 24.22 g/1 L MQH ₂ O)	31.25 ml
MQH ₂ O	68.05 ml

Recipes for Cell Stimulations

2 x Sample Buffer

	g/100 ml	
125 mM Tris	1.51	pH 6.8
4% SDS	4.00	
10% Mercaptoethanol (v/v)	10.0 ml	
20% Glycerol (w/v)	20.00	
0.04% Bromophenol blue	0.04	

Lysis Buffer

	g/100ml	
20 mM Tris (121.1 MW)	0.242	pH 7.5
137 mM NaCl (58.44 MW)	0.801	
10 mM NaF (41.99 MW), 500 mM stock: 420 mg/20 ml MQH ₂ O	2.00 ml	
1 mM EDTA (372.2 MW), 100 mM stock: 372.24 mg/10 ml MQH ₂ O	1.00 ml	
10% glycerol (w/v)	10.00	
1% NP-40 (w/v)	1.00	
1 mM Sodium orthovanadate (189.3 MW), 500 mM stock: 0.09195 g in 1.0 ml MQH ₂ O	0.2 μ l/ml	These reagents are added immediately prior to use
1 mM PMSF (174.2 MW), 500 mM stock: 0.0871 g in 1.0 ml DMSO	2 μ l/ml	
Aprotinin (2 mg/ml stock in MQH ₂ O)	2 μ l/ml	
Leupeptin (2 mg/ml stock in MQH ₂ O)	2 μ l/ml	
Pepstatin A (1 mg/ml stock in DMSO)	2 μ l/ml	

Recipes for In Vitro Lipid Kinase Assay

Lysis Buffer

	g/L	
20 mM Tris (121.1 MW)	0.242	pH 7.5
137 mM NaCl (58.44 MW)	0.801	
1 mM MgCl ₂ (203.3 MW), 100 mM stock: 203.3 mg/10 ml MQH ₂ O	1.0 ml	
1 mM CaCl ₂ (219.1 MW), 100 mM stock: 219.1 mg/10 ml MQH ₂ O	1.0 ml	
10% Glycerol (w/v)	10.00	
1% NP-40 (w/v)	1.00	
1 mM Sodium orthovanadate (189.3 MW), 500 mM stock: 0.09195 g in 1.0 ml MQH ₂ O	0.2 µl/ml	These reagents are added immediately prior to use
1 mM PMSF (174.2 MW), 500 mM stock: 0.0871 g in 1.0 ml DMSO	2 µl/ml	
Aprotinin (2 mg/ml stock in MQH ₂ O)	2 µl/ml	
Leupeptin (2 mg/ml stock in MQH ₂ O)	2 µl/ml	
Pepstatin A (1 mg/ml stock in DMSO)	2 µl/ml	

(note: the presence of MgCl₂ and CaCl₂ in place of NaF and EDTA found in ‘normal’ lysis buffer)

PBS + 1% NP-40

	g/L
NaCl	8.00
KCl	0.20
Na ₂ HPO ₄	1.15
KH ₂ PO ₄	0.20
NP-40 (w/v)	10.00
100 µM Sodium orthovanadate, 500 mM stock	0.2 µl/ml (add on day of use)

Tris/LiCl Buffer

	g/100 ml	
100 mM Tris	1.21	pH 7.4
5 mM LiCl (42.39 MW), 100 mM stock: 42.39 mg/10 ml MQH ₂ O	5.0 ml	
100 µM Sodium orthovanadate, 500 mM stock	0.2 µl/ml (add on day of use)	

TNE Buffer

	g/100 ml	
10 Mm Tris	0.121	pH 7.4
150 mM NaCl	0.88	
5 mM EDTA (372.24 MW), 100 mM stock: 372.24 mg/10 ml MQH ₂ O	5.0 ml	
100 µM Sodium orthovanadate, 500 mM stock	0.2 µl/ml (add on day of use)	

ATP Kinase Buffer

0.88 mM ATP, 8.8 mM stock: 8.8 µl (100 mM stock)/100 µl 10 mM Tris
 20 mM MgCl₂, 200 mM MgCl₂ stock: 20.33 g/20 mls MQH₂O
 5 µCi/5 µl [γ^{32} P]-ATP: e.g. (stock: 10 µCi/1 µl) for 10 samples require 50 µCi in 50 µl
 \therefore 5 µl γ -ATP + 5 µl 8.8 mM ATP + 5 µl 200 mM MgCl₂ + 35 µl 10 mM Tri *
 = 50 µCi/50 µl

* Note: make solution up to volume with 10 mM Tris (pH 7.4)

5 N HCl (100 ml)

43 ml concentrated HCl (36% pure, 1.18 g/ml density) + 57 ml MQH₂O

TLC Tank Resolving Buffer (120.3 ml)

Chloroform	60.0 ml
Methanol	47.0 ml
MQH ₂ O	11.3 ml
Ammonium hydroxide	2.0 ml

Recipes for Lipid Labelling

Media

Sterile phosphate-free DMEM (1 L) supplemented with sodium bicarbonate (49 ml of 7.5% stock) and 20 mM HEPES.

Primary Extraction Phase Solution

Chloroform	32.6 ml
Methanol	65.3 ml
MQH ₂ O	2.1 ml

10 µg/ml Folch Lipids (prepared just before use)

1 mg Folch lipids (from bovine brain, Folch fraction I lipids) in 100 ml chloroform

2.4 M HCl, 5 mM Tetrabutylammonium Sulphate (TBAS) (prepared just before use)

MQH ₂ O	39.7 ml
HCl (concentrated)	10.3 ml
TBAS	85 mg

0.1 M HCl, 5 mM EDTA (prepared just before use)

MQH ₂ O	47.07 ml
100 mM EDTA	2.50 ml
HCl (concentrated)	0.429 ml

Deacylation Solution

Methylamine (25% stock solution)	40 ml
Methanol	40 ml
n-butanol	10 ml

Petroleum Ether Solution

n-butanol	80 ml
Petroleum ether (bp 40 - 60°C)	16 ml
Ethyl formate	4 ml

1.25 M Ammonium Phosphate

$(\text{NH}_4)_2\text{HPO}_4$	165.08 g/1 L MQH ₂ O	pH 3.8 *
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* Note: pH adjusted with phosphoric acid (H₃PO₄). Buffer is filtered through 2 µm filters prior to use, and if not used immediately, degassed by re-filtering and bubbling through N₂ for 30 min before using.

Recipes for Concorde/MERLIN Calcium Assay***Physiological Salt Solution (PSS)******g/500 ml (10x stock) – pH 7.5***

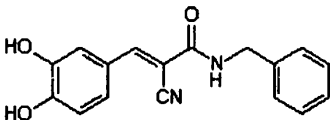
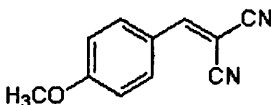
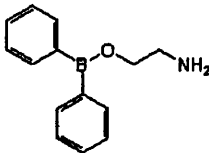
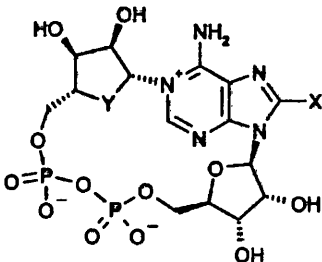
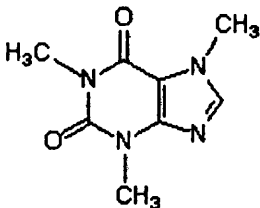
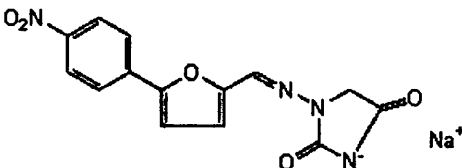
1.35 M Sodium chloride	39.45
54 mM Potassium chloride	2.00
20 mM Magnesium chloride	2.03
100 mM HEPES	11.91
2 mM* Calcium chloride	2 ml of 1 M stock/1 L*
10 mM* Glucose	1.8g/1 L*

*CaCl₂ and glucose are added to the 1 x PSS solution just prior to use, thus amounts and concentrations stated above pertain to 1 L of a 1 x PSS solution.

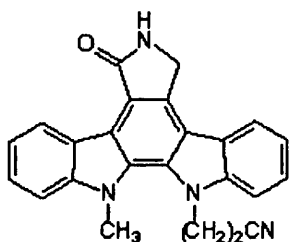
Fura-2-AM Stock

500 µg Fura-2 is dissolved in 99.8 µl pluronic F-127 acid (20% (w/v) DMSO) to give a 5 mM stock (1000 x working concentration). A working concentration is obtained by the addition of 10 µl 5 mM Fura-2 to 10 ml PSS. Cells are 'loaded' with 1 ml of this solution for 30 min in the dark.

6.2 Appendix 2: Inhibitor Pharmacology

NAME	STRUCTURE	TARGET	NOTES
AG490		JAK2/3	Inhibitor of JAK2 phosphorylation. Also inhibits EGFR kinase (IC_{50} = 100 nM) and JAK3. Light sensitive.
AG9		Inactive	An inactive compound that is used as a negative control for AG490 and other tyrphostins. Light sensitive.
2-APB		IP3R Ca^{2+}	Inhibits IP3-induced calcium release (IC_{50} = 42 μ M) without affecting IP3 binding to its receptor. Does not affect Ca^{2+} release from RR stores.
8-Br-cADPR		cADPR Ca^{2+}	Inhibits cADPR-mediated calcium release from caffeine-insensitive stores. Has no effect on the release of calcium from RR or IP3R calcium stores.
Caffeine		RR Ca^{2+}	Increases the calcium affinity of the calcium activation site of the RR at millimolar concentrations. In certain circumstances, caffeine will also induce the release of calcium from RR stores.
Dantrolene		RR Ca^{2+}	Inhibits binding of ryanodine but not of IP3. Binds to the ryanodine receptor and inhibits CICR.

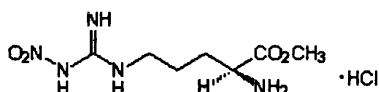
Gö6976



cPKC

Selective for the calcium-dependent PKCs (IC_{50} = 2-6 nM). Does not affect nPKC or aPKC activity. Inhibition is via competition with ATP and not protein substrate. Light sensitive.

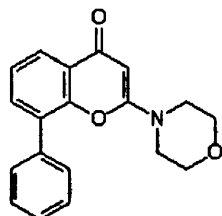
L-NAME



NOS

A stereospecific inhibitor of NO formation from L-arginine that is light sensitive. Competitive and a slowly reversible inhibitor of NOS (IC_{50} = 500 nM).

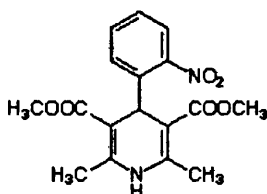
LY294002



PI3K

Inhibits (reversibly) PI3K (IC_{50} = 1.4 μ M), with greater potency for the class I PI3K isoforms over the class II members. Acts at the ATP-binding site of PI3K.

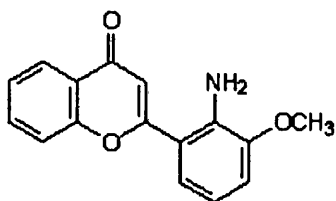
Nifedipine



NAADP/
L-type Ca^{2+}

Traditionally utilised as a blocker of L-type Ca^{2+} channels, but has been demonstrated to inhibit NAADP-mediated calcium release at higher concentrations.

PD98059



MEK

A light sensitive inhibitor of MAPK kinase (MEK). Acts by binding to the inactivated form of MEK, thereby preventing its phosphorylation by c-Raf or MEK kinase (IC_{50} = 2-7 μ M). May act via an allosteric mechanism.

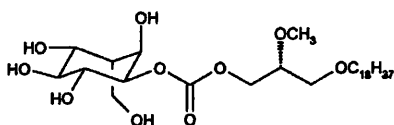
Pertussis Toxin

An exotoxin (holotoxin) consisting of 952 residues forming six subunits (S1-S5). Catalytic A subunit = S1; Cell binding B oligomer = S2, S3, S4 (x 2) and S5.

G α i

Catalyses ADP-ribosylation of guanine nucleotide-binding regulatory proteins G α i, G α o and G α T. Thus preventing the G protein heterotrimers from interacting with receptors. The α subunits remain in their GDP-bound state, unable to initiate pathway activation.

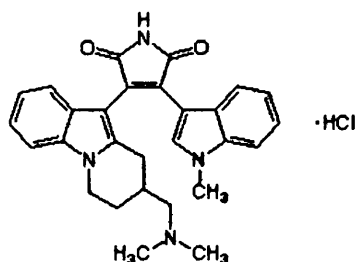
PKB inhibitor



PKB

Blocks PKB (IC₅₀ = 2 μ M) with more than 40-fold selectivity over PI3K (IC₅₀ = 83 μ M). Inhibition appears to be mediated through binding of the PKB-PH domain.

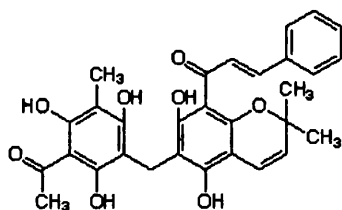
RO-32-0432



PKC

A selective cell permeable (light sensitive) PKC inhibitor (via ATP-binding competition, IC₅₀ = 9 - 100 nM) of both classical and novel isoforms (but with slightly better activity against the classical enzymes). Also inhibits GRK5.

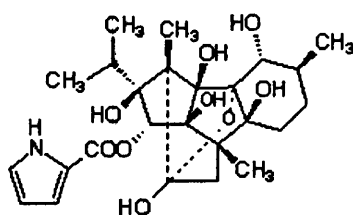
Rottlerin



PKC δ (?)

Initially identified as PKC δ isoform selective inhibitor (ATP-binding site, IC₅₀ = 3-6 μ M) with up to 10-fold selectivity over other isoforms. However, recent evidence queries this action.

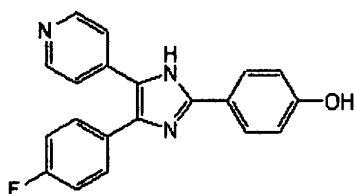
Ryanodine



RR Ca²⁺

Able to both inhibit and activate RRs. RR receptors have a low- and high-affinity site for ryanodine. Nanomolar concentrations sensitise the RR to CICR with micromolar amounts inhibiting.

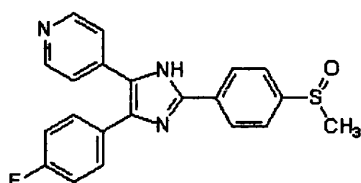
SB202190



p38

Potent cell-permeable inhibitor of p38 MAPK (IC_{50} = 16 nM (350 nM in cells)). Light sensitive. Binds ATP site in the enzyme's inactive state.

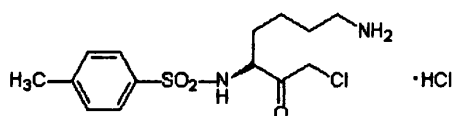
SB203580



p38

A light sensitive inhibitor of p38, with less potency than SB202190 (IC_{50} = 34 nM (600 nM in cells)). However, exhibits the same mode of action as SB202190.

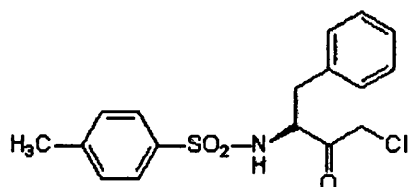
TLCK



NF- κ B?

Inhibits trypsin-like serine proteases. Blocks activation of NF- κ B and subsequent induction of the iNOS and COX2 genes. Effective concentration = 10 – 100 μ M. Blocks activation of p70S6K by all mitogens. Inactivates trypsin irreversibly. No effect on chymotrypsin.

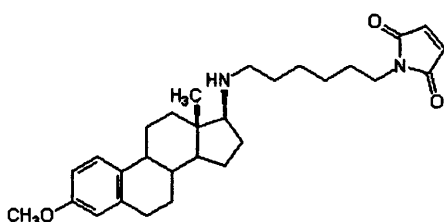
TPCK



NK- κ B?

Inhibits chymotrypsin-like serine proteases. Blocks activation of NF- κ B and subsequent induction of the iNOS and COX2 genes. Effective concentration = 10 – 100 μ M. Blocks activation of p70S6K by all mitogens. No effect on trypsin.

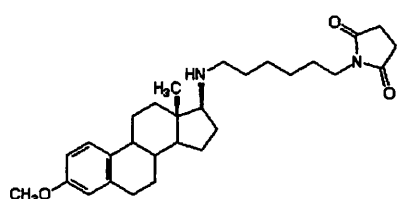
U73122



PLC (β)

An aminosteroid that inhibits G protein-mediated PLC activation (IC_{50} = 1 – 2 μ M in cells), possibly in a non-competitive manner.

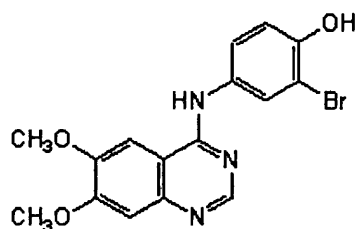
U73343



Inactive

The inactive analogue of U73122 and consequently used as a negative control.

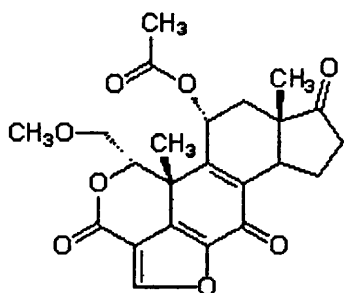
WHI-P154



JAK3

A potent, cell-permeable, specific inhibitor of JAK3 ($IC_{50} = 5.6 \mu M$). Has no effect on either JAK1 or JAK2. Inhibition is via binding to the catalytic site of JAK3. Light sensitive.

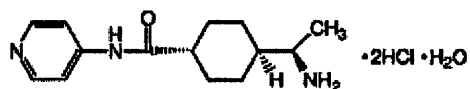
Wortmannin



PI3K

A potent and light-sensitive non-competitive and irreversible inhibitor of all classes of PI3K, with greater potency against the class I PI3K isoforms ($IC_{50} = 2 - 4 nM$). Structurally dissimilar to LY294002, and therefore useful for separating non-PI3K inhibitory activity from that of PI3K when used with LY294002. Activity is via covalent modification of the catalytic site.

Y-27632



ROCKI/II

A highly potent, cell-permeable and selective inhibitor of Rho-associated protein kinases (both ROCK I and II = 140 nM). Inhibition is competitive with respect to ATP. Light-sensitive.

6.3 Appendix 3: Inhibitor Practical Information

Inhibitor	Target	Concentration Range	Preincubation Time
2-APB	IP3R-mediated calcium release	2.25 – 75 μ M	1 hr
8-Br-cADPR	cADPR-mediated calcium release	50 μ M	1 hr
AG490	JAK2/3	Up to 100 μ M	1 hr
AG9	Negative control for AG490	Up to 100 μ M	1 hr
Caffeine*	RR-mediated calcium release	10 mM	30 min
Dantrolene	RR-mediated calcium release	100 μ M	1 hr
Gö6976	Classical PKC	30 – 300 nM	30 min
L-NAME	NOS	0.1 – 2 mM	1 hr
LY294002	PI3K	3 – 30 μ M	30 min
Nifedipine	NAADP and L-type calcium channels	50 μ M	1 hr
PKBi	PKB	10 μ M	3 hr
Anti-hMDC	MDC activity	10 μ g/ml	30 min
PD98059	MEK1/2	3 – 20 μ M	1 hr
Pertussis Toxin	G _o , G _q , G ₁₂ , G ₁₃	100ng/ml	16hrs
RO-32-0432	Classical and novel PKC	10 μ M	30 min
Rottlerin	PKC δ	10 μ M	1 hr
Ryanodine	RR-mediated calcium release	100 μ M	30 min
SB203580	p38-MAPK	10 μ M	1 hr
TLCK	Serine proteases (NF- κ B)	3 – 100 μ M	1 hr
TPCK	Serine proteases (NF- κ B)	3 – 100 μ M	1 hr
U73122	PLC	0.3 – 10 μ M	1 hr
U73343	Negative control for PLC	0.3 – 10 μ M	1 hr
WHI-P154	JAK3	50 μ M	1 hr
Wortmannin	PI3K	3 – 300 nM	15 min
Y-27632	ROCK	1 – 10 μ M	1 hr

* Caffeine utilised at an activating concentration.

6.4 Appendix 4: Antibody Affinities

Binding Strengths of Immunoglobulins to Protein A and Protein G

Species	Antibody	Protein A	Protein G
Human	IgG ₁	++	++
	IgG ₂	++	++
	IgG ₃	—	++
	IgG ₄	++	++
	IgM	+	—
	IgA	+	—
	IgE	+	—
	IgD	+	—
	Fab	+	+
	F(ab) ₂	+	+
	k light chain	—	—
	scFv	+	—
Mouse	IgG ₁	+	++
	IgG _{2a}	++	++
	IgG _{2b}	++	++
	IgG ₃	+	+
	IgM	+	—
Rat	IgG ₁	—	+
	IgG _{2a}	—	++
	IgG _{2b}	—	+
	IgG _{2c}	+	++
Rabbit	IgG	++	++
Horse	IgG	+	++
Pig	IgG	++	++
Sheep	IgG ₁	—	++
	IgG ₂	++	++
Goat	IgG	++	++
Chicken	IgY	—	—
Cow	IgG ₁	—	++
	IgG ₂	++	++

++ = Strong binding
 + = Moderate binding
 — = No binding

6.5 Appendix 5: Western Blotting Conditions

Antibody	% Bis-acrylamide Gel	Protein of Interest MW (kDa)	Blocking Conditions*	Primary Conditions*	Secondary Conditions
Pan-ERK 1/2 (Ab = 1 in 1000)	10	44/42	1% BSA in TBS + azide, overnight, rtp.	1% BSA + azide in TBST, 3hrs, rtp.	5 % milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000
Phospho ERK 1/2 (Thr 202/Tyr 204) (Ab = 1 in 1000)	10	44/42	1% BSA in TBS + azide, overnight, rtp.	1% BSA + azide in TBST, 3hrs, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000
Pan-GSK-3 α/β (Ab = 1 in 1000)	10	51/46	1% BSA in TBS + azide, overnight, rtp.	1% BSA + azide in TBST, 3hr, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000
Phospho GSK-3 α/β (Ser 21/9) (Ab = 1 in 1000)	10	51/46	1% BSA in TBS + azide, overnight, rtp.	1% BSA + azide in TBST, 3hr, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000
Phospho p90 ^{RSK} (Ser 380) (Ab = 1 in 1000)	7.5	90	5% milk in TBS, 3hrs, rtp.	3% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000
Pan-PKB (Ab = 1 in 1000)	10	60	5% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-goat Ab = 1 in 10000.
Phospho PKB (Thr 308) (Ab = 1 in 1000)	10	60	1% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000.
Phospho PKB (Ser 473) (Ab = 1 in 1000)	10	60	1% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000.
Phospho p38 (Thr 180/Tyr 182) (Ab = 1 in 1000)	10	38	5% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	1% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000.
Phospho MLC2 (Ser 19) (Ab = 1 in 1000)	15	18	5% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000.
Mystery Protein i.e. phospho FKHR (Ser 256) (Ab = 1 in 1000)	7.5 or 5	~ 200 (FKHR = 75)	5% milk in TBS, 2hr, rtp.	3% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000.
Phosphotyrosine (clone 4G10) (Ab = 1 in 1000)	Variable (7.5% for PYK2)	(PYK2 = 116)	1% BSA in TBS + azide, overnight, rtp.	1% BSA + azide in TBST, 3hr, rtp.	5% milk in TBST, 1hr, rtp. Anti-mouse Ig = 1 in 10000.
PTEN (Ab = 1 in 1000)	10	54	5% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000.
SHIP (Ab = 1 in 1000)	7.5	145	5% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-mouse Ig = 1 in 7000.
PI3K-C2 α (Ab = 1 in 1000)	7.5	170	5% milk in TBS, 1hr, rtp.	in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000.
PI3K C2 β (Ab = 1 in 1000)	7.5	165	5% milk in TBS, 1hr, rtp.	in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 10000.
PI3K-p110 δ (Ab = 1 in 1000)	7.5	110	1% BSA in TBS, 1hr, rtp.	in TBST, overnight, rtp.	1% milk in TBST, 1hr, rtp. Anti-goat Ig = 1 in 10000.
PI3K-p110 γ (Ab = 1 in 1000)	7.5	110	1% BSA in TBS, 1hr, rtp.	in TBST, overnight, rtp.	1% milk in TBST, 1hr, rtp. Anti-goat Ig = 1 in 10000.
PKC δ (Ab = 1 in 1000)	7.5	78	5% milk in TBS, 1hr, rtp.	1% BSA + azide in TBST, overnight, rtp.	5% milk in TBST, 1hr, rtp. Anti-rabbit Ig = 1 in 7000.

* azide is at 0.05%.

6.6 Appendix 6: Tables & Formulas

Metric prefixes.

Factor	Prefix	Symbol
10^{18}	Exa	E
10^{15}	Peta	P
10^{12}	Tetra	T
10^9	Giga	G
10^6	Mega	M
10^3	Kilo	k
10^{-3}	Milli	m
10^{-6}	Micro	μ
10^{-9}	Nano	n
10^{-12}	Pico	p
10^{-15}	Femto	f
10^{-18}	Atto	a

$$\begin{aligned}
 1 \text{ \AA} &= 10^{-10} \text{ m} \\
 (\text{\AA} \text{ngstr\AA} \text{m}) &10^{-8} \text{ cm} \\
 &10^{-4} \text{ }\mu\text{m} \\
 &10^{-1} \text{ nm}
 \end{aligned}$$

Centigrade \rightarrow Fahrenheit

$$^{\circ}\text{F} = 32 + (^{\circ}\text{C} \times 0.555)$$

Fahrenheit \rightarrow Centigrade

$$^{\circ}\text{C} = 0.555 \times (^{\circ}\text{F} - 32)$$

Greek alphabet.

A	α	Alpha
B	β	Beta
Γ	γ	Gamma
Δ	δ	Delta
E	ϵ	Epsilon
Z	ζ	Zeta
H	η	Eta
Θ	θ	Theta
I	ι	Iota
K	κ	Kappa
Λ	λ	Lambda
M	μ	Mu
N	ν	Nu
Ξ	ξ	Xi
O	\omicron	Omicron
Π	π	Pi
P	ρ	Rho
Σ	σ, ς	Sigma
T	τ	Tau
Y	υ	Upsilon
Φ	ϕ	Phi
X	χ	Chi
Ψ	ψ	Psi
Ω	ω	Omega

Centrifugal force \leftrightarrow revolutions per minute:

$$\text{rpm} = 1000 \sqrt{\frac{\text{RCF}}{1.12 \times R}}$$

$$\text{RCF} = 1.12 \times R \left(\frac{\text{rpm}}{1000} \right)^2$$

Where:

rpm = revolutions per minute

RCF = relative centrifugal force

R = radius of motor in mm

at room temperature.

Purity of DNA

Pure DNA: $A_{260}/A_{280} \geq 1.8$

An $A_{260}/A_{280} < 1.8$ indicates that the preparation is contaminated with proteins and aromatic substances (e.g. phenol).

An $A_{260}/A_{280} > 2.0$ indicates a possible contamination with RNA.

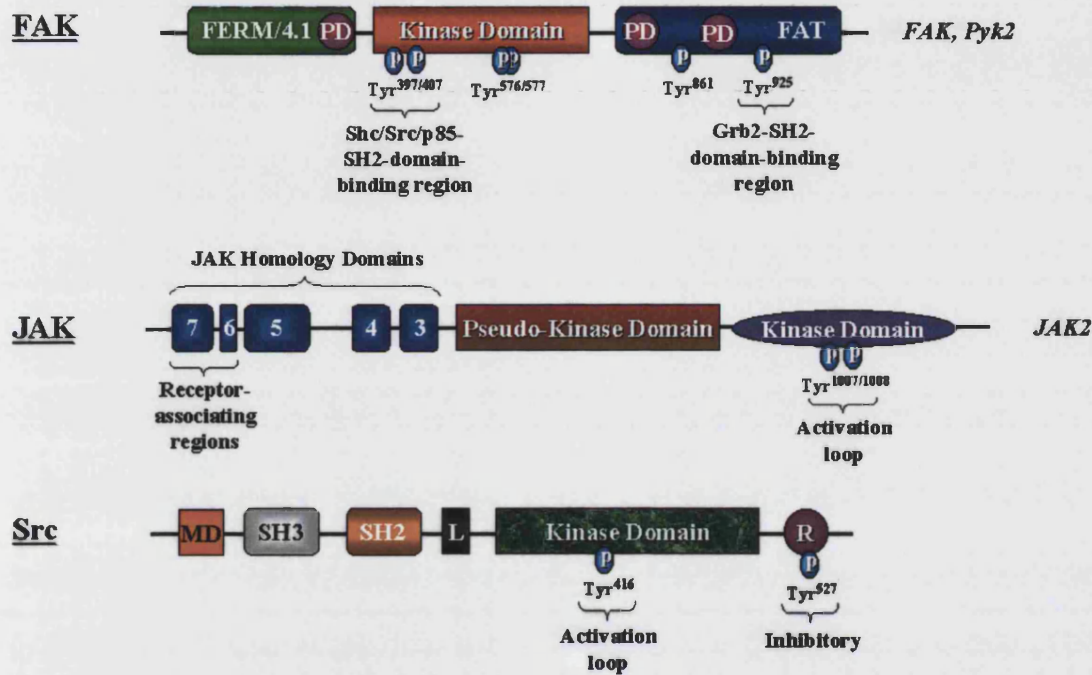
6.7 Appendix 7: Amino Acid Characteristics

Amino Acid	3 Letter Code	Single Letter Code	Chemical Properties				Compositor of Side Group (R)	
			MW (Da)	Genetic Code				
Alanine	Ala	A	89	GCU GCG	GCC	GCA	Hydrophobic	-CH ₃
Arginine	Arg	R	174	CGC CGG	CGC AGA	CGA AGG	Hydrophillic	-(CH ₃) ₃ -NH-CN ₂ -NH ₂
Asparagine	Asn	N	132	AAU	AAC		Hydrophillic	-CH ₂ -CONH ₂
Aspartic Acid (Aspartate)	Asp	D	133	GAU	GAC		Hydrophillic	-CH ₂ -COOH
Cysteine	Cys	C	121	UGU	UGC			-CH ₂ -SH
Glutamine	Gln	Q	146	CAA	CAG		Hydrophillic	-CH ₂ -CH ₂ -CONH ₂
Glutamic Acid (Glutamate)	Glu	E	147	GAA	GAG		Hydrophillic	-CH ₂ -CH ₂ -COOH
Glycine	Gly	G	75	GGU GGG	GGC	GGA		-H
Histidine	His	H	155	CAU	CAC		Hydrophillic	-CH ₂ -C ₃ N ₃ H ₄ ⁺ (C ₃ N ₃ H ₄ ⁺ = imidazole)
Isoleucine	Ile	I	131	AUU	AUC	AUA	Hydrophobic	-CH(CH ₃)-CH ₂ -CH ₃
Leucine	Leu	L	131	CUU CUG	CUC UUA	CUA UUG	Hydrophobic	-CH ₂ -CH(CH ₃) ₂
Lysine	Lys	K	146	AAA	AAG		Hydrophillic	-(CH ₂) ₄ -NH ₂
Methionine	Met	M	149	AUG			Hydrophobic	-CH ₂ -CH ₂ -S-CH ₃
Phenylalanine	Phe	F	165	UUU	UUC		Very Hydrophobic	-CH ₂ -C ₆ H ₆ (C ₆ H ₆ = benzene)
Proline	Pro	P	115	CCU CCG	CCC	CCA		-(CH ₂) ₃ ((CH ₂) ₃ = proline ring)
Serine	Ser	S	105	UCU UCG	UCC AGU	UCA AGC	Hydrophillic	-N-(CH ₂) ₃ -CH
Threonine	Thr	T	119	ACU ACG	ACC	ACA	Hydrophillic	-CH(CH ₃)-OH
Tryptophan	Trp	W	204	UGG			Very Hydrophobic	-CH ₂ -C-Ph-NH-CH (C-Ph-NH-CH = indole)
Tyrosine	Tyr	Y	181	UAU	UAC		Hydrophobic	-CH ₂ -C ₆ H ₆ -OH (C ₆ H ₆ = benzene)
Valine	Val	V	117	GUU GUG	GUC	GUA	Hydrophobic	-CH-(CH ₃) ₂

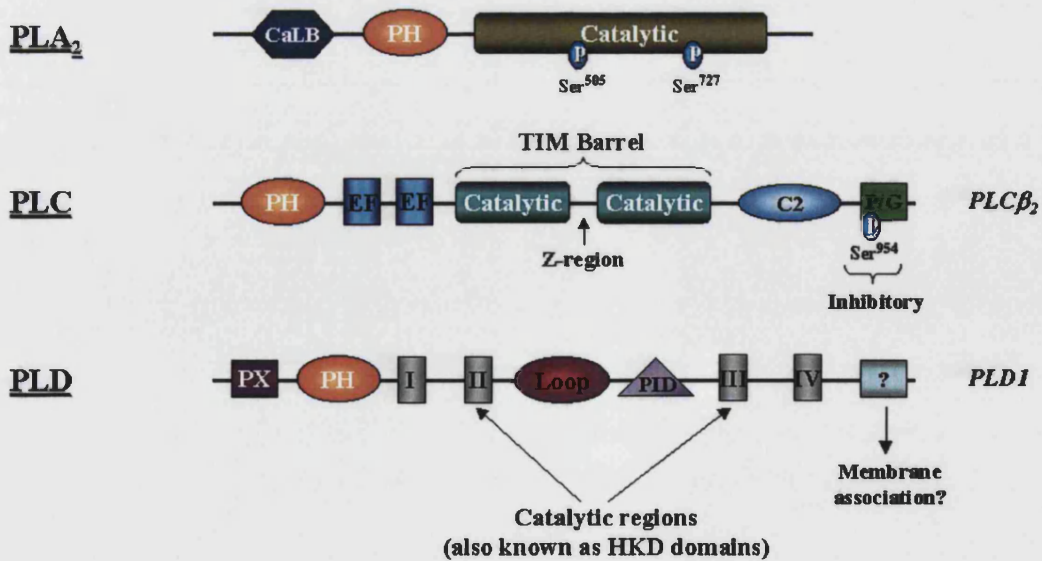
Basic amino acids are shown in blue, acidic amino acids in red, neutral amino acids in black, and amino acids with uncharged, polar residues in green. Average MW = 110 Da.

6.8 Appendix 8: Schematic Kinase Structures & Phosphorylation

Non-Receptor Tyrosine Kinases

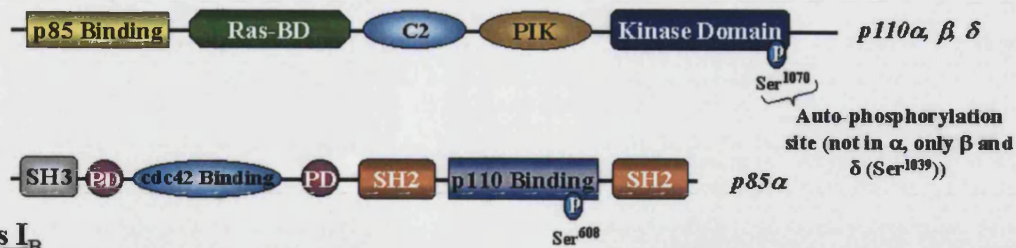


Phospholipases

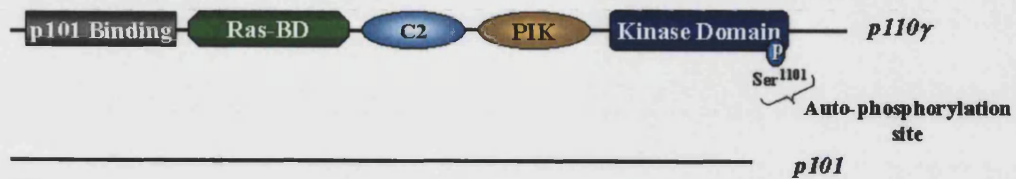


PI3K Isoforms

Class I_A



Class I_B



Class II

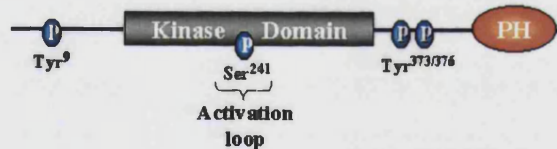


Class III

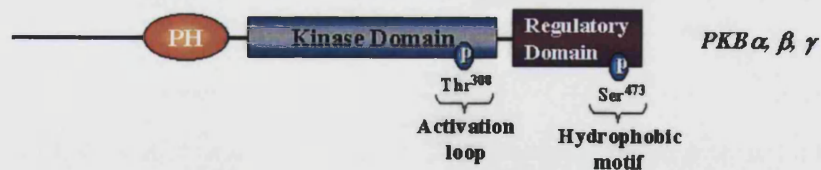


PI3K Effectors

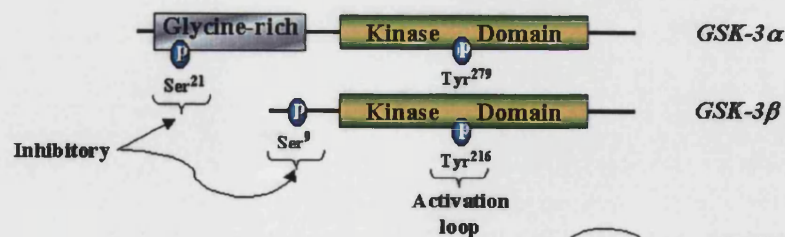
PDK-1



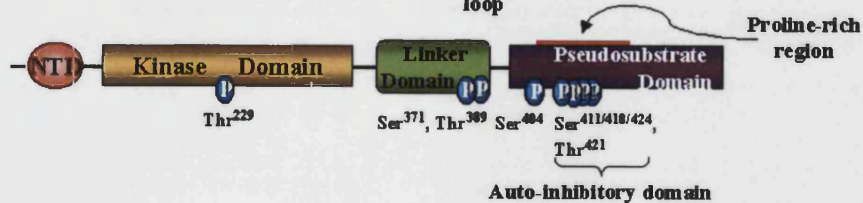
PKB



GSK-3



p70^{S6K}

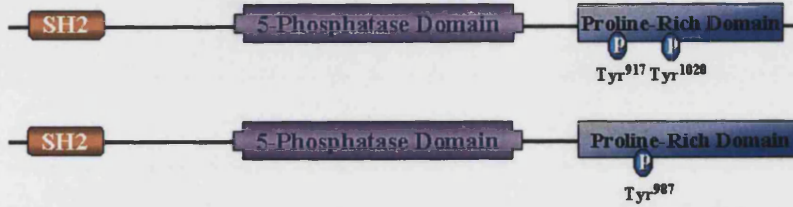


Phosphoinositide Phosphatases

PTEN



SHIP

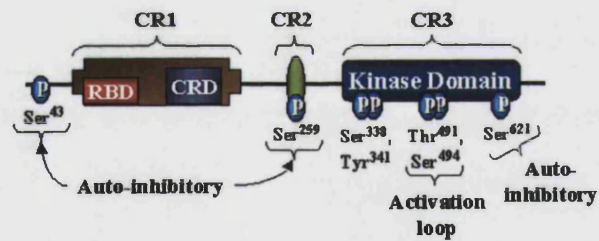


SHIP1

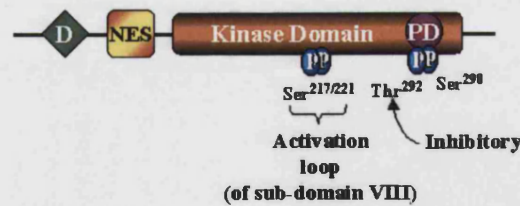
SHIP2

MAPK Pathway Proteins

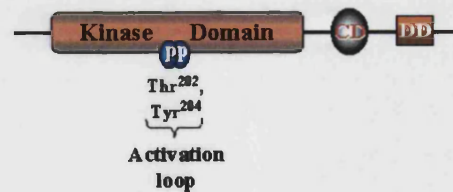
Raf



MEK

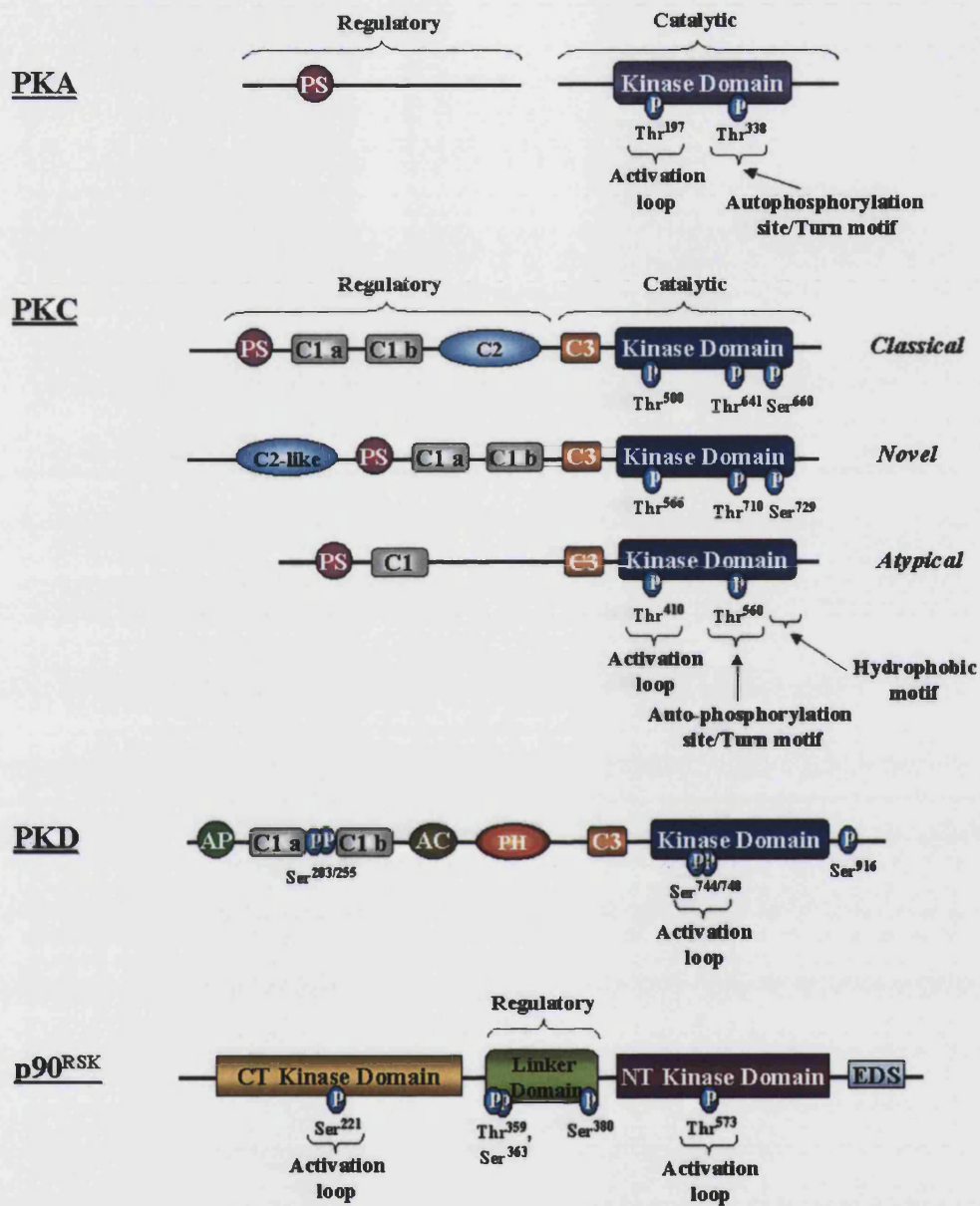


MAPK

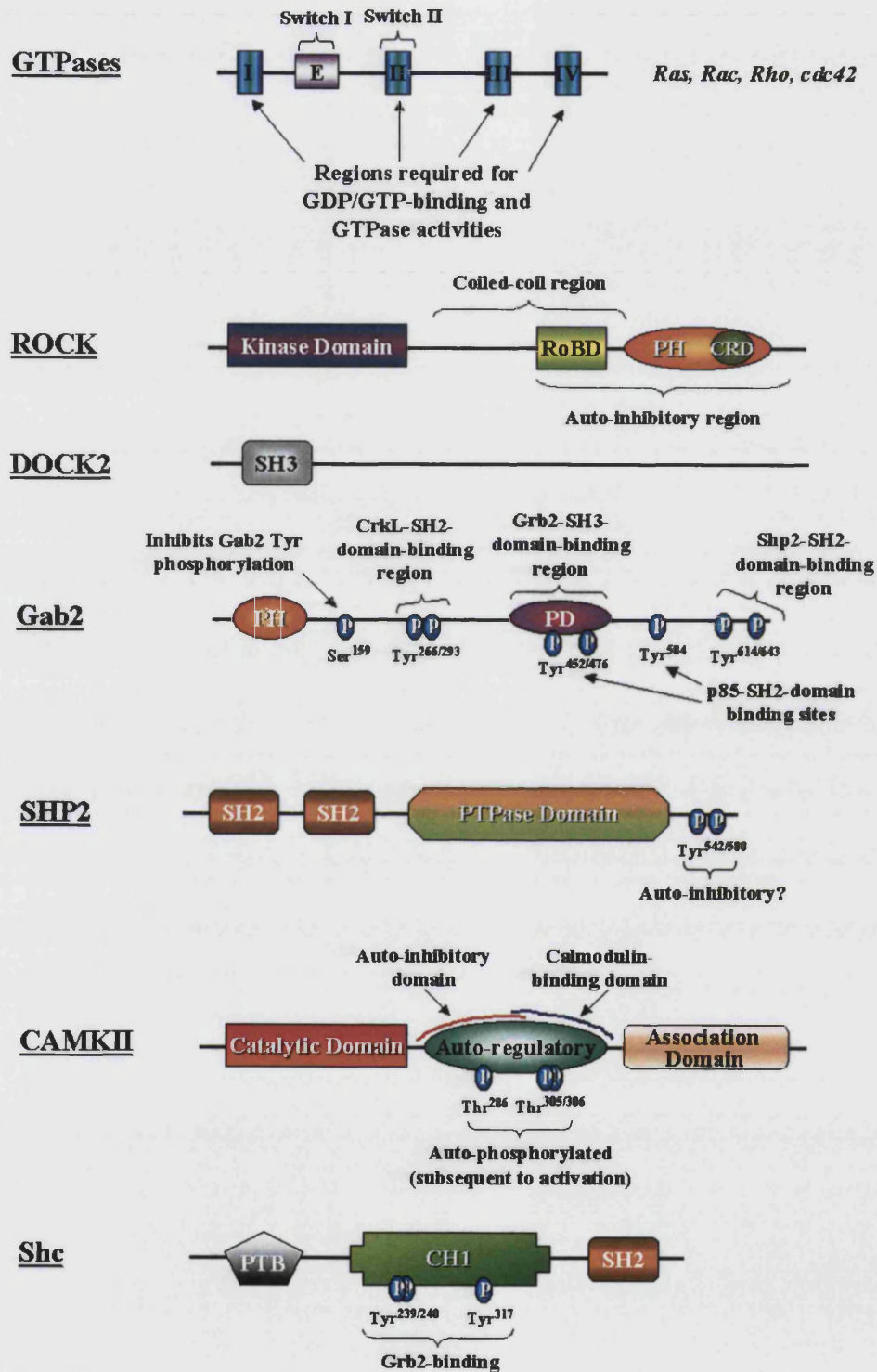


ERK, JNK, p38
Example = ERK1

Other AGC Kinases




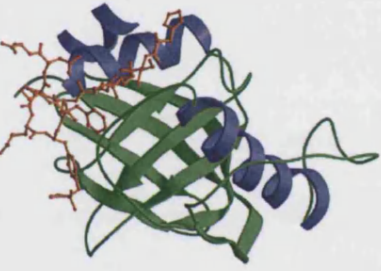
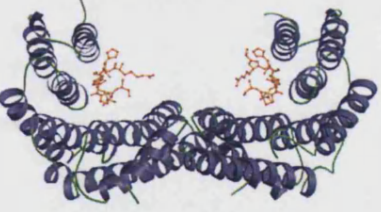
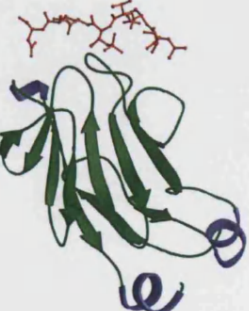
Other Signalling Proteins



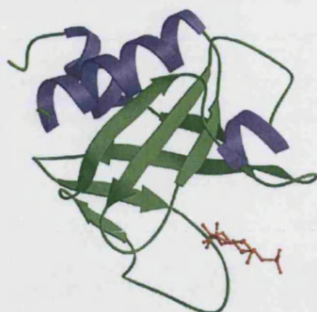
Diagrams display the domains present in each protein, with regions of interest highlighted along with residues that have been demonstrated to play an important role in the protein's function when phosphorylated (indicated with **P**).

Domain Abbreviations: AC, acidic domain; AP, alanine- and proline-rich domain; BD, binding domain; CaLB, calmodulin-binding domain; CD, common docking domain (also known as cytoplasmic retention sequence (CRS)); CH1, collagen homology domain 1; CR, conserved region; CRD, cysteine-rich domain; CT, C-terminal; D, docking domain; DD, dimerisation domain; E, effector-binding domain; EDS, ERK docking site; EF, EF hand domain; FAT, focal adhesion targeting domain; FERM/4.1, band 4.1/ezrin/radixin/moesin domain; L, linker domain; Loop, loop domain; MD, membrane-binding domain; NES, nuclear export sequence; NT, N-terminal; NTD, nuclear targeting domain; P/G, P/G box; PD, proline-rich domain; PH, pleckstrin-homology domain; PID, PIP₂-interacting domain; PS, pseudosubstrate; PIK, phosphatidylinositol kinase domain; PTB, phosphotyrosine-binding domain; PTPase, protein tyrosine phosphatase; PX, phox homology domain; R, regulatory domain; RBD, ras-binding domain; RoBD, rho-binding domain; SH2, src-homology 2 domain; SH3, src-homology 3 domain; ?, membrane-associating domain?

6.9 Appendix 9: Protein Interaction Domains

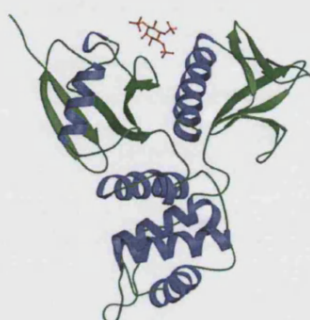
DOMAIN	BINDING SPECIFICITY OR ROLE	PROTEIN EXAMPLES	DESCRIPTION
SH2	Phospho-tyrosine	Btk, p85, Grb2, SHIP	Src-homology 2 (SH2) domains are modules of approximately 100 amino acids that bind specific phosphotyrosine-containing peptide motifs (e.g. p85 SH2 recognises pYXXM in target proteins), normally between 3-6 residues in size.
			
PTB	Phospho-tyrosine	Shc, IRS-1	Phosphotyrosine binding (PTB) domains are modules ranging from 100 – 150 amino acids that commonly bind Asn-Pro-X-Tyr (NPXpY) motifs (e.g. in the insulin receptor), but require more amino-terminal residues for high affinity binding (~ 12 residues in total).
			
14-3-3	Phospho-ser/thr	14-3-3 ζ	In mammals there are nine closely related 14-3-3 proteins that are 30 kDa in size and bind phosphoserine-containing motifs in binding partners (e.g. MEKK, c-Raf, PKC).
			
FHA	Phospho-ser/thr	AF-6, Rad 53	The Forkhead-Associated (FHA) domain is 65 – 100 amino acids long, containing several highly conserved key residues, which bind pTXXX peptides among others.
			

PH Phospholipid PKB, PLC, PDK



A wide variety of proteins that associate with membranes contain pleckstrin homology (PH) domains. Some PH domains bind specific phosphoinositides (e.g. PLC and $\text{PI}(4,5)\text{P}_2$, PKB and 3'-phosphoinositides) leading to relocation at membranes.

FERM Phospholipid Radixin, Ezrin, Moesin



Named after the initial 4 proteins the it was found in, the FERM domain is about 150 amino acids and is found in a variety of cytoskeletal-associated proteins. FERM domain is required $\text{PI}(4,5)\text{P}_2$ -regulated membrane binding of ERM. FERM domain of Radixin additionally recognises IP_3 as a ligand.

FYVE Phospholipid Hrs-2, FGD1, SARA



The FYVE (Fab-1, YOTB, Vac1 and EEA1) domain has been identified in more than 60 proteins and is a small, cysteine-rich Zn^{2+} binding domain of roughly 60 amino acids. It displays specificity for $\text{PI}(3)\text{P}$ and consequently has implicated FYVE domain-containing proteins downstream of $\text{PI}3\text{K}$.

ENTH Phospholipid Epsin1, AP180



The Epsin NH2-terminal homology (ENTH) domain is a membrane-binding motif of approximately 140 amino acids with selectivity for $\text{PI}(4,5)\text{P}_2$. ENTH domain containing proteins have been implicated as clathrin adaptors in endocytosis.

PX Phospholipid PLD1/2, PI3K



One of the most recently identified members of the phospholipid binding domains. Phox Homology (PX) domains are ~ 120 amino acids and function predominantly as D3-phosphorylated phosphoinositide [PI(3)P] binding modules, targeting the PX domain-containing proteins to the membranes.

C1 Phospholipid PKC isoforms, c-Raf



Domains that are enriched in cysteines of approximately 50 amino acids and are involved in the recruitment of proteins to the membrane. C1 domains generally bind DAG or phorbol esters.

C2 Phospholipid PKC β , Perforin 1



A region of 130 or so amino acids that is involved in binding phospholipids in a calcium-dependent or independent manner. Found in more than 100 proteins with functions ranging from signal transduction to vesicular trafficking.

WW Pro-rich sequence FBP-11, YAP



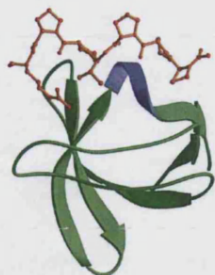
Small 38 – 40 amino acid residue modules that potentially bind overlapping sites with SH3 domains.

GYF Pro-rich sequence CD2BP2, Smy2



Glycine-tyrosine-phenylalanine (GYF) is a proline-rich peptide-binding domain but is structurally unrelated to SH3 or WW domains. CD2BP2 binds a proline-rich peptide sequence in tail region of CD2.

SH3 Pro-rich sequence p85, Grb2, Src



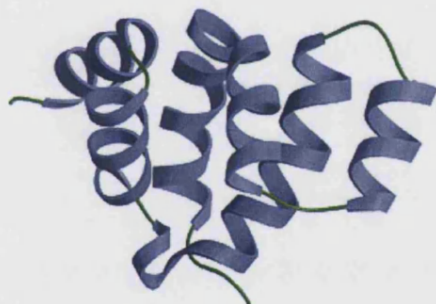
Found in numerous proteins, the Src-homology 3 (SH3) domains bind to proline-rich peptides that form a left-handed poly-Pro type II helix, with the minimal consensus Pro-X-X-Pro. Src SH3 can bind a motif within p85 PI3K.

EVH1 Pro-rich sequence WASP, MENA



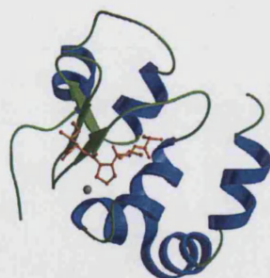
A module of ~110 amino acids found in some scaffolding proteins involved in the control of the actin cytoskeleton. Ena/Vasp Homology domain 1 (EVH1) recognise proline-rich motifs in components of the cytoskeleton, such as vinculin and Zyxin.

DD Apoptosis NF- κ B p100, DAPK



Death domains (DD) are 80 – 100 residue motifs involved in apoptosis signalling. Found in both receptors (e.g. TNF family) and cytoplasmic proteins, they heterodimerize with death domains of distinct proteins (e.g. FADD adaptor) via electrostatic interactions.

BIR Apoptosis IAP-1, Survivin

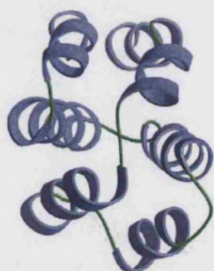


The Baculovirus IAP Repeat (BIR) domain is a ~70 amino acid zinc-binding module. It has been demonstrated to be necessary for the interaction of Inhibitors of Apoptosis (IAP) proteins with diverse proapoptotic factors, promoting cell survival.

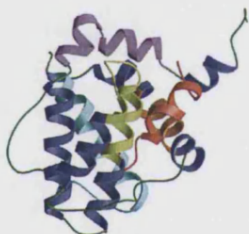
DED	Apoptosis	FADD, Procaspase-10	The Death Effector Domain (DED) is found in inactive procaspases and proteins that regulate caspase activation. DEDs recruit procaspases into complexes with adaptor molecules containing a second DED that is directly associated with activated TNF receptors.
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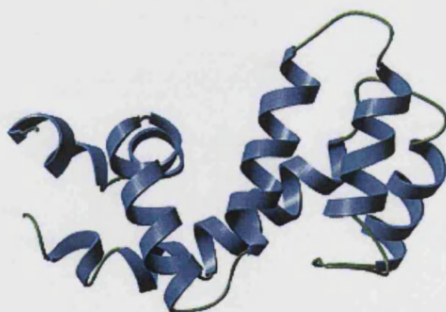
CARD	Apoptosis	Procaspase-2, APAF-1	Caspase Recruitment Domains (CARDs) act in a similar manner to that of the DED domains. They are modules of 90 – 100 amino acids, and like DEDs, mediate the heterodimerisation of adaptor proteins and procaspases via their respective CARDs.
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BH1-4	Apoptosis	Bcl-2, BAX, Bcl-xL	Bcl-2 Homology (BH1-4) domains are found in proteins that inhibit apoptosis. These proteins can form homo- or heterodimers via these various BH domains found in both interacting molecules.
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RGS	Protein interactions (known specificity)	p115RhoGEF, RGS4	The RGS (Regulator of G protein Signalling) is typically about 120 amino acids in length. RGS domains act allosterically by stabilizing the transition intermediate of the GTP binding pocket of the alpha subunit of heterotrimeric G proteins. This results in the acceleration of the intrinsic GTPase activity of that alpha subunit.
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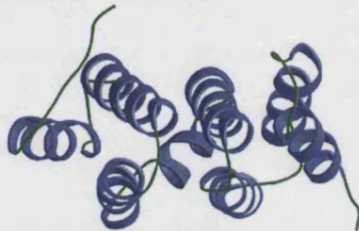


PDZ Protein interactions (known specificity) FAP, PSD-95, Dsh



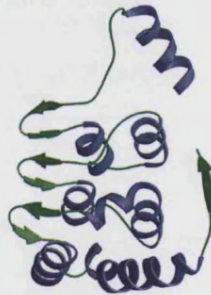
PDZ domains bind to 4 – 5 residues of their target proteins (normally transmembrane receptors or ion channels) with high affinity. The consensus sequence is found in the C-terminus of target proteins and contains frequently contain a hydrophobic molecule (e.g. Val or Ile), and the second and third residues determine specificity. Additionally, PDZ domains are able to heterodimerize with other PDZ domains.

VHS Protein interactions (known specificity) Hrs, STAM, Vps27



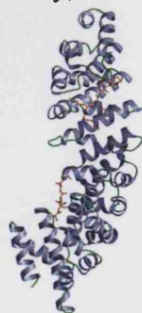
Vps27p, Hrs and Stam (VHS) domain is approximately 150 amino acids in size and bind target molecules via other domains, including Hrs with Hrs FYVE domain. The domain may play a role in receptor tyrosine kinases signalling and endocytosis.

ANK Protein interactions (variable specificity) DAPK, Shank



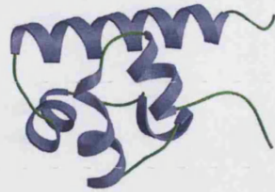
Although ANK domains have been found in more than 600 proteins, to date it is unknown what constitutes an ANK domain target. Targets of ANK domains include p53 and CDK6, and the cytoskeletal protein Ankyrin is almost entirely composed of these short 33 amino acid repeats.

ARM Protein interactions (variable specificity) β -Catenin, APC protein



The Armadillo (ARM) 40 amino acid repeat is named after the *Drosophila* protein it was found in (β -catenin is the human homologue). It has subsequently been found in more than 100 proteins with no common features among the target proteins identified.

SAM Protein interactions (variable specificity) Slp-76, CNK, Scm



The Sterile Alpha Motif (SAM) has been implicated in the formation of homo- and heterotypic oligomers.

WD40 Protein interactions (variable specificity) G β , Prp4, Sec13



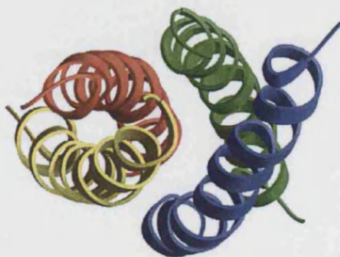
The common functional theme of WD40 domains is to serve as a stable platform to which proteins can bind. WD40 proteins do not have catalytic activity unlike the non-WD40 propeller family of proteins. A notable protein that contains the WD40 domain is that of the G-protein β chain that allows it to bind the G-protein α, γ -chains.

BRCT Protein interactions (variable specificity) DNA ligase III, BARD BRCA1 C-terminus (BRCA1)


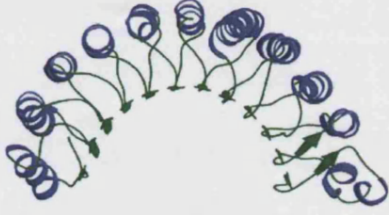




domains are found mostly in proteins involved in cell cycle checkpoint functions responsive to DNA damage.

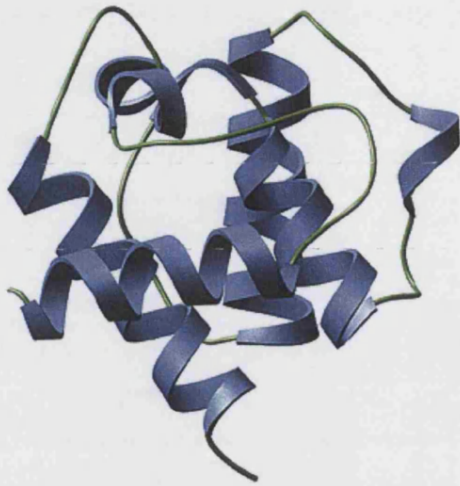
CC Protein interactions (variable specificity) Stat1, Fos, Jun



Coiled-coils (CC) domains function as oligomerisation domains for a number of proteins. It is thought that 5% of proteins encoded in sequenced genomes contain coiled-coils (two or more α -helices wrapped around each other with a superhelical twist). CCs can form homo- or heterotypic oligomers.

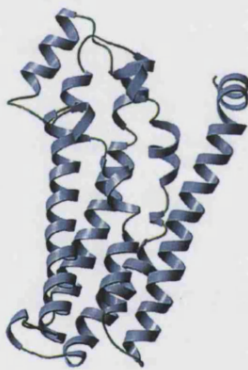
LIM	Protein interactions (variable specificity)	LIMK, Enigma	Identified initially in 3 developmentally regulated transcription factors Lin-1, Isl-1 and Mec-3 (LIM) and now identified in more than 300 proteins, the LIM domain is a zinc-binding, cysteine-rich motif consisting of 2 tandemly repeated zinc fingers. LIM domain containing proteins have a wide variety of functions.
			
LRR	Protein interactions (variable specificity)	Skp2, TAP, Rna1p	Leucine-Rich repeats (LRR) are 22 – 28 amino acid motifs involved in protein – protein interactions and form crescent-shaped structures acting as a scaffold for protein interactions.
			
EF-hand	Calcium	Calmodulin, Calcineurin	Approximately 40 residues in size and is involved in binding intracellular calcium. Structural EF-hand domains seem to play a role in buffering intracellular calcium levels.
			
ADF	Cytoskeleton	Cofilin, Drebrin, ADF	The Actin-Depolymerising Factor (ADF) domain is found in proteins involved in F-actin severing. ADF proteins bind to monomeric and filamentous actin and sever actin filaments leading to faster actin turnover.
			

CH Cytoskeleton Calponin, Vav



The calponin homology (CH) domain is present in cytoskeletal and signal transduction proteins. Tandem CH (type 1 & 2) domains, at the amino terminus, bind F-actin and cross-link filaments into bundles and networks. Type 3 CH domains are found in proteins that regulate muscle contraction, such as calponin, as well as in signalling proteins, such as Vav, and may not interact directly with actin. Instead they act as regulatory domains or protein-protein interaction scaffolds to modulate the activity of proteins.

DH RhoGTPase RhoGEFs, Tiam-1



Dbl homology (DH) or RhoGEF domains induce Rho family GTPases to displace GDP, thereby effectively activating the Rho GTPase by allowing binding to GTP. The DH domain is usually preceded by a PH domain, which appears to enhance catalytic efficiency. Also found in Rac/cdc42 binding proteins such as Tiam1 and Dbl.

Structural diagrams were obtained from Cell Signaling Technologies (www.cellsignal.com).

7

BIBLIOGRAPHY

7. BIBLIOGRAPHY

Abi-Younes,S., Si-Tahar,M., and Luster,A.D. (2001). The CC chemokines MDC and TARC induce platelet activation via CCR4. *Thromb. Res.* *101*, 279-289.

Adachi,T., Stafford,S., Kayaba,H., Chihara,J., and Alam,R. (2003). Myosin light chain kinase mediates eosinophil chemotaxis in a mitogen- activated protein kinase-dependent manner. *J. Allergy Clin. Immunol.* *111*, 113-116.

Adachi,T., Vita,R., Sannohe,S., Stafford,S., Alam,R., Kayaba,H., and Chihara,J. (2001). The functional role of rho and rho-associated coiled-coil forming protein kinase in eotaxin signaling of eosinophils. *J. Immunol.* *167*, 4609-4615.

Agace,W.W., Roberts,A.I., Wu,L., Greineder,C., Ebert,E.C., and Parker,C.M. (2000). Human intestinal lamina propria and intraepithelial lymphocytes express receptors specific for chemokines induced by inflammation. *Eur. J. Immunol.* *30*, 819-826.

Al Aoukaty,A., Rolstad,B., and Maghazachi,A.A. (1999). Recruitment of pleckstrin and phosphoinositide 3-kinase gamma into the cell membranes, and their association with G beta gamma after activation of NK cells with chemokines. *J. Immunol.* *162*, 3249-3255.

Alessi,D.R., Andjelkovic,M., Caudwell,B., Cron,P., Morrice,N., Cohen,P., and Hemmings,B.A. (1996). Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* *15*, 6541-6551.

Alessi,D.R., Cuenda,A., Cohen,P., Dudley,D.T., and Saltiel,A.R. (1995). PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J. Biol. Chem.* *270*, 27489-27494.

Alessi,D.R., Deak,M., Casamayor,A., Caudwell,F.B., Morrice,N., Norman,D.G., Gaffney,P., Reese,C.B., MacDougall,C.N., Harbison,D., Ashworth,A., and Bownes,M. (1997a). 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. *Curr. Biol.* *7*, 776-789.

Alessi,D.R., James,S.R., Downes,C.P., Holmes,A.B., Gaffney,P.R., Reese,C.B., and Cohen,P. (1997b). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. *Curr. Biol.* *7*, 261-269.

Ali,H., Ahamed,J., Hernandez-Munain,C., Baron,J.L., Krangel,M.S., and Patel,D.D. (2000). Chemokine production by G protein-coupled receptor activation in a human mast cell line: roles of extracellular signal-regulated kinase and NFAT. *J. Immunol.* *165*, 7215-7223.

Allen,V., Swigart,P., Cheung,R., Cockcroft,S., and Katan,M. (1997). Regulation of inositol lipid-specific phospholipase cdelta by changes in Ca²⁺ ion concentrations. *Biochem. J.* *327 (Pt 2)*, 545-552.

Alon,R., Chen,S., Fuhlbrigge,R., Puri,K.D., and Springer,T.A. (1998). The kinetics and shear threshold of transient and rolling interactions of L-selectin with its ligand on leukocytes. *Proc. Natl. Acad. Sci. U. S. A* *95*, 11631-11636.

Alon,R., Kassner,P.D., Carr,M.W., Finger,E.B., Hemler,M.E., and Springer,T.A. (1995). The integrin VLA-4 supports tethering and rolling in flow on VCAM-1. *J. Cell Biol.* *128*, 1243-1253.

Alteraifi,A.M. and Zhelev,D.V. (1997). Transient increase of free cytosolic calcium during neutrophil motility responses. *J. Cell Sci.* *110 (Pt 16)*, 1967-1977.

- Altura,B.M. and Gebrewold,A. (2002). Inhibitor of nuclear factor-Kappa B activation attenuates venular constriction, leukocyte rolling-adhesion and microvessel rupture induced by ethanol in intact rat brain microcirculation: relation to ethanol-induced brain injury. *Neurosci. Lett.* 334, 21-24.
- Amano,M., Fukata,Y., and Kaibuchi,K. (2000). Regulation and functions of Rho-associated kinase. *Exp. Cell Res.* 261, 44-51.
- Amano,M., Ito,M., Kimura,K., Fukata,Y., Chihara,K., Nakano,T., Matsuura,Y., and Kaibuchi,K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J. Biol. Chem.* 271, 20246-20249.
- Amara,A., Lorthioir,O., Valenzuela,A., Magerus,A., Thelen,M., Montes,M., Virelizier,J.L., Delepierre,M., Baleux,F., Lortat-Jacob,H., and Arenzana-Seisdedos,F. (1999). Stromal cell-derived factor-1alpha associates with heparan sulfates through the first beta-strand of the chemokine. *J. Biol. Chem.* 274, 23916-23925.
- Amin,A.R., Ichigotani,Y., Oo,M.L., Biswas,M.H., Yuan,H., Huang,P., Mon,N.N., and Hamaguchi,M. (2003). The PLC-PKC cascade is required for IL-1beta-dependent Erk and Akt activation: their role in proliferation. *Int. J. Oncol.* 23, 1727-1731.
- Andrew,D.P., Chang,M.S., McNinch,J., Wathen,S.T., Rihane,M., Tseng,J., Spellberg,J.P., and Elias,C.G., III (1998). STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. *J. Immunol.* 161, 5027-5038.
- Andrew,D.P., Ruffing,N., Kim,C.H., Miao,W., Heath,H., Li,Y., Murphy,K., Campbell,J.J., Butcher,E.C., and Wu,L. (2001). C-C chemokine receptor 4 expression defines a major subset of circulating nonintestinal memory T cells of both Th1 and Th2 potential. *J. Immunol.* 166, 103-111.
- Annunziato,F., Romagnani,P., Cosmi,L., Lazzeri,E., and Romagnani,S. (2001). Chemokines and lymphopoiesis in human thymus. *Trends Immunol.* 22, 277-281.
- Appleman,L.J., Tzachanis,D., Grader-Beck,T., van Puijenbroek,A.A., and Boussiotis,V.A. (2001). Helper T cell anergy: from biochemistry to cancer pathophysiology and therapeutics. *J. Mol. Med.* 78, 673-683.
- Arai,H. and Charo,I.F. (1996). Differential regulation of G-protein-mediated signaling by chemokine receptors. *J. Biol. Chem.* 271, 21814-21819.
- Arbuzova,A., Schmitz,A.A., and Vergeres,G. (2002). Cross-talk unfolded: MARCKS proteins. *Biochem. J.* 362, 1-12.
- Arcaro,A., Zvelebil,M.J., Wallasch,C., Ullrich,A., Waterfield,M.D., and Domin,J. (2000). Class II phosphoinositide 3-kinases are downstream targets of activated polypeptide growth factor receptors. *Mol. Cell Biol.* 20, 3817-3830.
- Arndt,P.G., Suzuki,N., Avdi,N.J., Malcolm,K.C., and Worthen,G.S. (2003). Lipopolysaccharide induced c-Jun NH2-terminal kinase activation in human neutrophils: Role of phosphatidylinositol-3 kinase and Syk mediated pathways. *J. Biol. Chem.*
- Ascher-Landsberg,J., Saunders,T., Elovitz,M., and Phillippe,M. (1999). The effects of 2-aminoethoxydiphenyl borate, a novel inositol 1,4, 5-trisphosphate receptor modulator on myometrial contractions. *Biochem. Biophys. Res. Commun.* 264, 979-982.
- Ashida,N., Arai,H., Yamasaki,M., and Kita,T. (2001a). Differential signaling for MCP-1-dependent integrin activation and chemotaxis. *Ann. N. Y. Acad. Sci.* 947, 387-389.
- Ashida,N., Arai,H., Yamasaki,M., and Kita,T. (2001b). Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. *J. Biol. Chem.* 276, 16555-16560.

- Asojo,O.A., Boulegue,C., Hoover,D.M., Lu,W., and Lubkowski,J. (2003). Structures of thymus and activation-regulated chemokine (TARC). *Acta Crystallogr. D. Biol. Crystallogr.* *59*, 1165-1173.
- Aspenstrom,P. (1999a). Effectors for the Rho GTPases. *Curr. Opin. Cell Biol.* *11*, 95-102.
- Aspenstrom,P. (1999b). The Rho GTPases have multiple effects on the actin cytoskeleton. *Exp. Cell Res.* *246*, 20-25.
- Astoul,E., Edmunds,C., Cantrell,D.A., and Ward,S.G. (2001). PI 3-K and T-cell activation: limitations of T-leukemic cell lines as signaling models. *Trends Immunol.* *22*, 490-496.
- Avraham,H., Park,S.Y., Schinkmann,K., and Avraham,S. (2000). RAFTK/Pyk2-mediated cellular signalling. *Cell Signal.* *12*, 123-133.
- Baba,M., Nishimura,O., Kanzaki,N., Okamoto,M., Sawada,H., Iizawa,Y., Shiraishi,M., Aramaki,Y., Okonogi,K., Ogawa,Y., Meguro,K., and Fujino,M. (1999). A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. *Proc. Natl. Acad. Sci. U. S. A* *96*, 5698-5703.
- Bacon,K.B., Flores-Romo,L., Life,P.F., Taub,D.D., Premack,B.A., Arkinstall,S.J., Wells,T.N., Schall,T.J., and Power,C.A. (1995). IL-8-induced signal transduction in T lymphocytes involves receptor-mediated activation of phospholipases C and D. *J. Immunol.* *154*, 3654-3666.
- Bacon,K.B., Schall,T.J., and Dairaghi,D.J. (1998). RANTES activation of phospholipase D in Jurkat T cells: requirement of GTP-binding proteins ARF and RhoA. *J. Immunol.* *160*, 1894-1900.
- Baggiolini,M. (2001). Chemokines in pathology and medicine. *J. Intern. Med.* *250*, 91-104.
- Baggiolini,M., Dewald,B., and Moser,B. (1997). Human chemokines: an update. *Annu. Rev. Immunol.* *15*, 675-705.
- Baier,G. (2003). The PKC gene module: molecular biosystematics to resolve its T cell functions. *Immunol. Rev.* *192*, 64-79.
- Balendran,A., Casamayor,A., Deak,M., Paterson,A., Gaffney,P., Currie,R., Downes,C.P., and Alessi,D.R. (1999). PDK1 acquires PDK2 activity in the presence of a synthetic peptide derived from the carboxyl terminus of PRK2. *Curr. Biol.* *9*, 393-404.
- Ballif,B.A., Shimamura,A., Pae,E., and Blenis,J. (2001). Disruption of 3-phosphoinositide-dependent kinase 1 (PDK1) signaling by the anti-tumorigenic and anti-proliferative agent n-alpha-tosyl-l-phenylalanyl chloromethyl ketone. *J. Biol. Chem.* *276*, 12466-12475.
- Barandier,C., Ming,X.F., Rusconi,S., and Yang,Z. (2003). PKC is required for activation of ROCK by RhoA in human endothelial cells. *Biochem. Biophys. Res. Commun.* *304*, 714-719.
- Bardi,G., Niggli,V., and Loetscher,P. (2003). Rho kinase is required for CCR7-mediated polarization and chemotaxis of T lymphocytes. *FEBS Lett.* *542*, 79-83.
- Bargatze,R.F., Jutila,M.A., and Butcher,E.C. (1995). Distinct roles of L-selectin and integrins alpha 4 beta 7 and LFA-1 in lymphocyte homing to Peyer's patch-HEV in situ: the multistep model confirmed and refined. *Immunity.* *3*, 99-108.
- Barna,J.B. and Kew,R.R. (1995). Inhibition of neutrophil chemotaxis by protease inhibitors. Differential effect of inhibitors of serine and thiol proteases. *Inflammation* *19*, 561-574.
- Bauer,B., Jenny,M., Fresser,F., Uberall,F., and Baier,G. (2003). AKT1/PKBalpha is recruited to lipid rafts and activated downstream of PKC isoforms in CD3-induced T cell signaling. *FEBS Lett.* *541*, 155-162.

- Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., and Schall, T.J. (1997). A new class of membrane-bound chemokine with a CX3C motif. *Nature* 385, 640-644.
- Bellacosa, A., Testa, J.R., Staal, S.P., and Tsichlis, P.N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* 254, 274-277.
- Berg, E.L., McEvoy, L.M., Berlin, C., Bargatze, R.F., and Butcher, E.C. (1993). L-selectin-mediated lymphocyte rolling on MAdCAM-1. *Nature* 366, 695-698.
- Berin, M.C., Dwinell, M.B., Eckmann, L., and Kagnoff, M.F. (2001a). Production of MDC/CCL22 by human intestinal epithelial cells. *Am. J. Physiol Gastrointest. Liver Physiol* 280, G1217-G1226.
- Berin, M.C., Eckmann, L., Broide, D.H., and Kagnoff, M.F. (2001b). Regulated production of the T helper 2-type T-cell chemoattractant TARC by human bronchial epithelial cells in vitro and in human lung xenografts. *Am. J. Respir. Cell Mol. Biol.* 24, 382-389.
- Berlin, C., Bargatze, R.F., Campbell, J.J., Von Andrian, U.H., Szabo, M.C., Hasslen, S.R., Nelson, R.D., Berg, E.L., Erlandsen, S.L., and Butcher, E.C. (1995). alpha 4 integrins mediate lymphocyte attachment and rolling under physiologic flow. *Cell* 80, 413-422.
- Bernardini, G., Hedrick, J., Sozzani, S., Luini, W., Spinetti, G., Weiss, M., Menon, S., Zlotnik, A., Mantovani, A., Santoni, A., and Napolitano, M. (1998). Identification of the CC chemokines TARC and macrophage inflammatory protein-1 beta as novel functional ligands for the CCR8 receptor. *Eur. J. Immunol.* 28, 582-588.
- Berridge, M.J., Bootman, M.D., and Lipp, P. (1998). Calcium—a life and death signal. *Nature* 395, 645-648.
- Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat. Rev. Mol. Cell Biol.* 4, 517-529.
- Berridge, M.J., Lipp, P., and Bootman, M.D. (2000). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1, 11-21.
- Biedermann, T., Schwarzler, C., Lametschwandner, G., Thoma, G., Carballido-Perrig, N., Kund, J., de Vries, J.E., Rot, A., and Carballido, J.M. (2002). Targeting CLA/E-selectin interactions prevents CCR4-mediated recruitment of human Th2 memory cells to human skin in vivo. *Eur. J. Immunol.* 32, 3171-3180.
- Birle, D., Bottini, N., Williams, S., Huynh, H., deBelle, J., Adamson, E., and Mustelin, T. (2002). Negative feedback regulation of the tumor suppressor PTEN by phosphoinositide-induced serine phosphorylation. *J. Immunol.* 169, 286-291.
- Blair, L.A. and Marshall, J. (1997). IGF-1 modulates N and L calcium channels in a PI 3-kinase-dependent manner. *Neuron* 19, 421-429.
- Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., and Springer, T.A. (1996). The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 382, 829-833.
- Bleul, C.C., Schultze, J.L., and Springer, T.A. (1998). B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. *J. Exp. Med.* 187, 753-762.
- Bochner, B.S., Bickel, C.A., Taylor, M.L., MacGlashan, D.W., Jr., Gray, P.W., Raport, C.J., and Godiska, R. (1999). Macrophage-derived chemokine induces human eosinophil chemotaxis in a CC chemokine receptor 3- and CC chemokine receptor 4-independent manner. *J. Allergy Clin. Immunol.* 103, 527-532.
- Bochner, B.S., Hudson, S.A., Xiao, H.Q., and Liu, M.C. (2003). Release of both CCR4-active and CXCR3-active chemokines during human allergic pulmonary late-phase reactions. *J. Allergy Clin. Immunol.* 112, 930-934.

- Boehncke,W.H. and Schon,M.P. (2003). Interfering with leukocyte rolling--a promising therapeutic approach in inflammatory skin disorders? *Trends Pharmacol. Sci.* 24, 49-52.
- Bonacchi,A., Romagnani,P., Romanelli,R.G., Efsen,E., Annunziato,F., Lasagni,L., Francalanci,M., Serio,M., Laffi,G., Pinzani,M., Gentilini,P., and Marra,F. (2001). Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes. *J. Biol. Chem.* 276, 9945-9954.
- Bonecchi,R., Bianchi,G., Bordignon,P.P., D'Ambrosio,D., Lang,R., Borsatti,A., Sozzani,S., Allavena,P., Gray,P.A., Mantovani,A., and Sinigaglia,F. (1998). Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 187, 129-134.
- Bonello,M.R. and Khachigian,L.M. (2003). Fibroblast growth factor-2 represses platelet-derived growth factor receptor-alpha transcription via ERK1/2-dependent Sp1 phosphorylation and an atypical Cis-acting element in the proximal PDGFR-alpha promoter. *J. Biol. Chem.*
- Bootman,M.D., Berridge,M.J., and Roderick,H.L. (2002). Calcium signalling: more messengers, more channels, more complexity. *Curr. Biol.* 12, R563-R565.
- Bootman,M.D., Collins,T.J., Peppiatt,C.M., Prothero,L.S., Mackenzie,L., de Smet,P., Travers,M., Tovey,S.C., Seo,J.T., Berridge,M.J., Ciccolini,F., and Lipp,P. (2001). Calcium signalling--an overview. *Semin. Cell Dev. Biol.* 12, 3-10.
- Borchers,M.T., Ansay,T., DeSalle,R., Daugherty,B.L., Shen,H., Metzger,M., Lee,N.A., and Lee,J.J. (2002). In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration. *J. Leukoc. Biol.* 71, 1033-1041.
- Bowman,E.P., Campbell,J.J., Soler,D., Dong,Z., Manlongat,N., Picarella,D., Hardy,R.R., and Butcher,E.C. (2000). Developmental switches in chemokine response profiles during B cell differentiation and maturation. *J. Exp. Med.* 191, 1303-1318.
- Brahmbhatt,A.A. and Klemke,R.L. (2003). ERK and RhoA differentially regulate pseudopodia growth and retraction during chemotaxis. *J. Biol. Chem.* 278, 13016-13025.
- Brazil,D.P. and Hemmings,B.A. (2001). Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem. Sci.* 26, 657-664.
- Breithaupt,T.B., Vazquez,A., Baez,I., and Eylar,E.H. (1996). The suppression of T cell function and NF(kappa)B expression by serine protease inhibitors is blocked by N-acetylcysteine. *Cell Immunol.* 173, 124-130.
- Brock,C., Schaefer,M., Reusch,H.P., Czupalla,C., Michalke,M., Spicher,K., Schultz,G., and Numborg,B. (2003). Roles of G beta gamma in membrane recruitment and activation of p110 gamma/p101 phosphoinositide 3-kinase gamma. *J. Cell Biol.* 160, 89-99.
- Brown,R.A., Domin,J., Arcaro,A., Waterfield,M.D., and Shepherd,P.R. (1999). Insulin activates the alpha isoform of class II phosphoinositide 3-kinase. *J. Biol. Chem.* 274, 14529-14532.
- Brundage,R.A., Fogarty,K.E., Tuft,R.A., and Fay,F.S. (1991). Calcium gradients underlying polarization and chemotaxis of eosinophils. *Science* 254, 703-706.
- Brundage,R.A., Fogarty,K.E., Tuft,R.A., and Fay,F.S. (1993). Chemotaxis of newt eosinophils: calcium regulation of chemotactic response. *Am. J. Physiol* 265, C1527-C1543.
- Burridge,K. and Wennerberg,K. (2004). Rho and Rac take center stage. *Cell* 116, 167-179.

Cairns,P., Okami,K., Halachmi,S., Halachmi,N., Esteller,M., Herman,J.G., Jen,J., Isaacs,W.B., Bova,G.S., and Sidransky,D. (1997). Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res.* 57, 4997-5000.

Caloca,M.J., Zugaza,J.L., Vicente-Manzanares,M., Sanchez-Madrid,F., and Bustelo,X.R. (2004). F-Actin-dependent translocation of the Rap1 GDP/GTP exchange factor RasGRP2. *J. Biol. Chem.*

Campbell,D.J., Kim,C.H., and Butcher,E.C. (2003). Chemokines in the systemic organization of immunity. *Immunol. Rev.* 195, 58-71.

Campbell,J.J., Brightling,C.E., Symon,F.A., Qin,S., Murphy,K.E., Hodge,M., Andrew,D.P., Wu,L., Butcher,E.C., and Wardlaw,A.J. (2001). Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. *J. Immunol.* 166, 2842-2848.

Campbell,J.J. and Butcher,E.C. (2000). Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr. Opin. Immunol.* 12, 336-341.

Campbell,J.J., Haraldsen,G., Pan,J., Rottman,J., Qin,S., Ponath,P., Andrew,D.P., Warnke,R., Ruffing,N., Kassam,N., Wu,L., and Butcher,E.C. (1999a). The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 400, 776-780.

Campbell,J.J., Pan,J., and Butcher,E.C. (1999b). Cutting edge: developmental switches in chemokine responses during T cell maturation. *J. Immunol.* 163, 2353-2357.

Cantley,L.C. (2002). The phosphoinositide 3-kinase pathway. *Science* 296, 1655-1657.

Cantrell,D. (2002). Protein kinase B (Akt) regulation and function in T lymphocytes. *Semin. Immunol.* 14, 19-26.

Cantrell,D.A. (2001). Phosphoinositide 3-kinase signalling pathways. *J. Cell Sci.* 114, 1439-1445.

Cara,D.C., Kaur,J., Forster,M., McCafferty,D.M., and Kubes,P. (2001). Role of p38 mitogen-activated protein kinase in chemokine-induced emigration and chemotaxis in vivo. *J. Immunol.* 167, 6552-6558.

Carafoli,E. (2002). Calcium signaling: a tale for all seasons. *Proc. Natl. Acad. Sci. U. S. A* 99, 1115-1122.

Carey,K.D., Dillon,T.J., Schmitt,J.M., Baird,A.M., Holdorf,A.D., Straus,D.B., Shaw,A.S., and Stork,P.J. (2000). CD28 and the tyrosine kinase Ick stimulate mitogen-activated protein kinase activity in T cells via inhibition of the small G protein Rap1. *Mol. Cell Biol.* 20, 8409-8419.

Carnevale,K.A. and Cathcart,M.K. (2003). Protein kinase C beta is required for human monocyte chemotaxis to MCP-1. *J. Biol. Chem.* 278, 25317-25322.

Cascieri,M.A. and Springer,M.S. (2000). The chemokine/chemokine-receptor family: potential and progress for therapeutic intervention. *Curr. Opin. Chem. Biol.* 4, 420-427.

Cenni,V., Doppler,H., Sonnenburg,E.D., Maraldi,N., Newton,A.C., and Toker,A. (2002). Regulation of novel protein kinase C epsilon by phosphorylation. *Biochem. J.* 363, 537-545.

Chalasani,S.H., Baribaud,F., Coughlan,C.M., Sunshine,M.J., Lee,V.M., Doms,R.W., Littman,D.R., and Raper,J.A. (2003). The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. *J. Neurosci.* 23, 4601-4612.

Chan,T.O., Rittenhouse,S.E., and Tsichlis,P.N. (1999). AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* 68, 965-1014.

- Chandrasekar,B., Bysani,S., and Mummidi,S. (2003a). CXCL16 signals via Gi, PI3 kinase, Akt, Ikappa B kinase and nuclear factor-kappa B, and induces cell-cell adhesion and aortic smooth muscle cell proliferation. *J. Biol. Chem.*
- Chandrasekar,B., Mummidi,S., Perla,R.P., Bysani,S., Dulin,N.O., Liu,F., and Melby,P.C. (2003b). Fractalkine (CX3CL1) stimulated by nuclear factor kappaB (NF-kappaB)-dependent inflammatory signals induces aortic smooth muscle cell proliferation through an autocrine pathway. *Biochem. J.* 373, 547-558.
- Chantry,D., Romagnani,P., Raport,C.J., Wood,C.L., Epp,A., Romagnani,S., and Gray,P.W. (1999). Macrophage-derived chemokine is localized to thymic medullary epithelial cells and is a chemoattractant for CD3(+), CD4(+), CD8(low) thymocytes. *Blood* 94, 1890-1898.
- Chantry,D., Vojtek,A., Kashishian,A., Holtzman,D.A., Wood,C., Gray,P.W., Cooper,J.A., and Hockstra,M.F. (1997). p110delta, a novel phosphatidylinositol 3-kinase catalytic subunit that associates with p85 and is expressed predominantly in leukocytes. *J. Biol. Chem.* 272, 19236-19241.
- Chardin,P., Paris,S., Antonny,B., Robineau,S., Beraud-Dufour,S., Jackson,C.L., and Chabre,M. (1996). A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. *Nature* 384, 481-484.
- Chavrier,P. and Goud,B. (1999). The role of ARF and Rab GTPases in membrane transport. *Curr. Opin. Cell Biol.* 11, 466-475.
- Cherla,R.P. and Ganju,R.K. (2001). Stromal cell-derived factor 1 alpha-induced chemotaxis in T cells is mediated by nitric oxide signaling pathways. *J. Immunol.* 166, 3067-3074.
- Chodniewicz,D. and Zhelev,D.V. (2003a). Chemoattractant receptor-stimulated F-actin polymerization in the human neutrophil is signaled by 2 distinct pathways. *Blood* 101, 1181-1184.
- Chodniewicz,D. and Zhelev,D.V. (2003b). Novel pathways of F-actin polymerization in the human neutrophil. *Blood* 102, 2251-2258.
- Choe,H., Farzan,M., Sun,Y., Sullivan,N., Rollins,B., Ponath,P.D., Wu,L., Mackay,C.R., LaRosa,G., Newman,W., Gerard,N., Gerard,C., and Sodroski,J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135-1148.
- Chou,M.M., Hou,W., Johnson,J., Graham,L.K., Lee,M.H., Chen,C.S., Newton,A.C., Schaffhausen,B.S., and Toker,A. (1998). Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr. Biol.* 8, 1069-1077.
- Christian,S.L., Sims,P.V., and Gold,M.R. (2002). The B cell antigen receptor regulates the transcriptional activator beta-catenin via protein kinase C-mediated inhibition of glycogen synthase kinase-3. *J. Immunol.* 169, 758-769.
- Chung,C.W., Cooke,R.M., Proudfoot,A.E., and Wells,T.N. (1995). The three-dimensional solution structure of RANTES. *Biochemistry* 34, 9307-9314.
- Chung,C.Y., Funamoto,S., and Firtel,R.A. (2001). Signaling pathways controlling cell polarity and chemotaxis. *Trends Biochem. Sci.* 26, 557-566.
- Chvatchko,Y., Hoogewerf,A.J., Meyer,A., Alouani,S., Juillard,P., Buser,R., Conquet,F., Proudfoot,A.E., Wells,T.N., and Power,C.A. (2000). A key role for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock. *J. Exp. Med.* 191, 1755-1764.
- Cinamon,G., Shinder,V., and Alon,R. (2001). Shear forces promote lymphocyte migration across vascular endothelium bearing apical chemokines. *Nat. Immunol.* 2, 515-522.
- Claing,A., Chen,W., Miller,W.E., Vitale,N., Moss,J., Premont,R.T., and Lefkowitz,R.J. (2001). beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis. *J. Biol. Chem.* 276, 42509-42513.

- Clemetson, K.J., Clemetson, J.M., Proudfoot, A.E., Power, C.A., Baggiolini, M., and Wells, T.N. (2000). Functional expression of CCR1, CCR3, CCR4, and CXCR4 chemokine receptors on human platelets. *Blood* 96, 4046-4054.
- Clore, G.M., Appella, E., Yamada, M., Matsushima, K., and Gronenborn, A.M. (1989). Determination of the secondary structure of interleukin-8 by nuclear magnetic resonance spectroscopy. *J. Biol. Chem.* 264, 18907-18911.
- Coffer, P.J. and Woodgett, J.R. (1991). Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur. J. Biochem.* 201, 475-481.
- Cohen, P. and Frame, S. (2001). The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* 2, 769-776.
- Conroy, D.M., Jopling, L.A., Lloyd, C.M., Hodge, M.R., Andrew, D.P., Williams, T.J., Pease, J.E., and Sabroe, I. (2003). CCR4 blockade does not inhibit allergic airways inflammation. *J. Leukoc. Biol.* 74, 558-563.
- Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J.Y., Butcher, E.C., and Laudanna, C. (2000). Chemokines trigger immediate beta2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity*. 13, 759-769.
- Cox, E.A., Sastry, S.K., and Huttenlocher, A. (2001). Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. *Mol. Biol. Cell* 12, 265-277.
- Coxon, P.Y., Rane, M.J., Uriarte, S., Powell, D.W., Singh, S., Butt, W., Chen, Q., and McLeish, K.R. (2003). MAPK-activated protein kinase-2 participates in p38 MAPK-dependent and ERK-dependent functions in human neutrophils. *Cell Signal.* 15, 993-1001.
- Cramer, L.P. (1999). Role of actin-filament disassembly in lamellipodium protrusion in motile cells revealed using the drug jasplakinolide. *Curr. Biol.* 9, 1095-1105.
- Cristillo, A.D. and Bierer, B.E. (2003). Regulation of CXCR4 expression in human T lymphocytes by calcium and calcineurin. *Mol. Immunol.* 40, 539-553.
- Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789.
- Crump, M.P., Gong, J.H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J.L., Baggiolini, M., Sykes, B.D., and Clark-Lewis, I. (1997). Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. *EMBO J.* 16, 6996-7007.
- Cuevas, B.D., Lu, Y., Mao, M., Zhang, J., LaPushin, R., Siminovitch, K., and Mills, G.B. (2001). Tyrosine phosphorylation of p85 relieves its inhibitory activity on phosphatidylinositol 3-kinase. *J. Biol. Chem.* 276, 27455-27461.
- Cui, Y., Galione, A., and Terrar, D.A. (1999). Effects of photoreleased cADP-ribose on calcium transients and calcium sparks in myocytes isolated from guinea-pig and rat ventricle. *Biochem. J.* 342 (Pt 2), 269-273.
- Cullen, P.J., Cozier, G.E., Banting, G., and Mellor, H. (2001). Modular phosphoinositide-binding domains--their role in signalling and membrane trafficking. *Curr. Biol.* 11, R882-R893.
- Cullen, P.J. and Lockyer, P.J. (2002). Integration of calcium and Ras signalling. *Nat. Rev. Mol. Cell Biol.* 3, 339-348.
- Curnock, A.P., Logan, M.K., and Ward, S.G. (2002). Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. *Immunology* 105, 125-136.

Curnock,A.P., Sotsios,Y., Wright,K.L., and Ward,S.G. (2003). Optimal Chemotactic Responses of Leukemic T Cells to Stromal Cell- Derived Factor-1 Requires the Activation of Both Class IA and IB Phosphoinositide 3-Kinases. *J. Immunol.* *170*, 4021-4030.

Cyster,J.G. (1999). Chemokines and cell migration in secondary lymphoid organs. *Science* *286*, 2098-2102.

D'Ambrosio,D., Iellem,A., Bonecchi,R., Mazzeo,D., Sozzani,S., Mantovani,A., and Sinigaglia,F. (1998). Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. *J. Immunol.* *161*, 5111-5115.

Damaj,B.B., McColl,S.R., Mahana,W., Crouch,M.F., and Naccache,P.H. (1996). Physical association of Gi2alpha with interleukin-8 receptors. *J. Biol. Chem.* *271*, 12783-12789.

Damen,J.E., Liu,L., Rosten,P., Humphries,R.K., Jefferson,A.B., Majerus,P.W., and Krystal,G. (1996). The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetrakisphosphate and phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase. *Proc. Natl. Acad. Sci. U. S. A* *93*, 1689-1693.

Davies,S.P., Reddy,H., Caivano,M., and Cohen,P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* *351*, 95-105.

DAVIS,B.J. (1964). DISC ELECTROPHORESIS. II. METHOD AND APPLICATION TO HUMAN SERUM PROTEINS. *Ann. N. Y. Acad. Sci.* *121*, 404-427.

Davis,C.B., Dikic,I., Unutmaz,D., Hill,C.M., Arthos,J., Siani,M.A., Thompson,D.A., Schlessinger,J., and Littman,D.R. (1997). Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J. Exp. Med.* *186*, 1793-1798.

de Lavareille,A., Roufosse,F., Schandene,L., Stordeur,P., Cogan,E., and Goldman,M. (2001). Clonal Th2 cells associated with chronic hypereosinophilia: TARC- induced CCR4 down-regulation in vivo. *Eur. J. Immunol.* *31*, 1037-1046.

Dean,M., Carrington,M., Winkler,C., Huttley,G.A., Smith,M.W., Allikmets,R., Goedert,J.J., Buchbinder,S.P., Vittinghoff,E., Gomperts,E., Donfield,S., Vlahov,D., Kaslow,R., Saah,A., Rinaldo,C., Detels,R., and O'Brien,S.J. (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* *273*, 1856-1862.

Dell'Acqua,M.L., Faux,M.C., Thorburn,J., Thorburn,A., and Scott,J.D. (1998). Membrane-targeting sequences on AKAP79 bind phosphatidylinositol-4, 5-bisphosphate. *EMBO J.* *17*, 2246-2260.

Dempsey,E.C., Newton,A.C., Mochly-Rosen,D., Fields,A.P., Reyland,M.E., Insel,P.A., and Messing,R.O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. *Am. J. Physiol Lung Cell Mol. Physiol* *279*, L429-L438.

Deng,H., Liu,R., Ellmeier,W., Choe,S., Unutmaz,D., Burkhart,M., di Marzio,P., Marmon,S., Sutton,R.E., Hill,C.M., Davis,C.B., Peiper,S.C., Schall,T.J., Littman,D.R., and Landau,N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* *381*, 661-666.

Di Cristofano,A., Pesce,B., Cordon-Cardo,C., and Pandolfi,P.P. (1998). Pten is essential for embryonic development and tumour suppression. *Nat. Genet.* *19*, 348-355.

Di,C., V, Strippoli,R., Bizzarri,C., Troiani,G., Cervellera,M.N., Gloaguen,I., Colagrande,A., Cattozzo,E.M., Pagliei,S., Santoni,A., Colotta,F., Mainiero,F., and Bertini,R. (2004). Key role of proline-rich tyrosine kinase 2 in interleukin-8 (CXCL8/IL-8)-mediated human neutrophil chemotaxis. *Immunology* *111*, 407-415.

Dieu,M.C., Vanbervliet,B., Vicari,A., Bridon,J.M., Oldham,E., Ait-Yahia,S., Briere,F., Zlotnik,A., Lebecque,S., and Caux,C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J. Exp. Med.* *188*, 373-386.

- Dikic,I., Dikic,I., and Schlessinger,J. (1998). Identification of a new Pyk2 isoform implicated in chemokine and antigen receptor signaling. *J. Biol. Chem.* 273, 14301-14308.
- Dillon,T.J., Karpitski,V., Wetzels,S.A., Parker,D.C., Shaw,A.S., and Stork,P.J. (2003). Ectopic B-Raf expression enhances extracellular signal-regulated kinase (ERK) signaling in T cells and prevents antigen-presenting cell-induced anergy. *J. Biol. Chem.* 278, 35940-35949.
- DiNitto,J.P., Cronin,T.C., and Lambright,D.G. (2003). Membrane recognition and targeting by lipid-binding domains. *Sci. STKE.* 2003, re16.
- Djordjevic,S. and Driscoll,P.C. (2002). Structural insight into substrate specificity and regulatory mechanisms of phosphoinositide 3-kinases. *Trends Biochem. Sci.* 27, 426-432.
- Doble,B.W. and Woodgett,J.R. (2003). GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* 116, 1175-1186.
- Dominguez,I. and Green,J.B. (2001). Missing links in GSK3 regulation. *Dev. Biol.* 235, 303-313.
- Donaldson,J.G. (2003). Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *J. Biol. Chem.* 278, 41573-41576.
- Donaldson,J.G. and Klausner,R.D. (1994). ARF: a key regulatory switch in membrane traffic and organelle structure. *Curr. Opin. Cell Biol.* 6, 527-532.
- Doornbos,R.P., Theelen,M., van der Hoeven,P.C., van Blitterswijk,W.J., Verkleij,A.J., and van Bergen en Henegouwen PM (1999). Protein kinase Czeta is a negative regulator of protein kinase B activity. *J. Biol. Chem.* 274, 8589-8596.
- Doranz,B.J., Rucker,J., Yi,Y., Smyth,R.J., Samson,M., Peiper,S.C., Parmentier,M., Collman,R.G., and Doms,R.W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta- chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85, 1149-1158.
- Dower,N.A., Stang,S.L., Bottorff,D.A., Ebinu,J.O., Dickie,P., Ostergaard,H.L., and Stone,J.C. (2000). RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat. Immunol.* 1, 317-321.
- Dragic,T., Litwin,V., Allaway,G.P., Martin,S.R., Huang,Y., Nagashima,K.A., Cayanan,C., Maddon,P.J., Koup,R.A., Moore,J.P., and Paxton,W.A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC- CKR-5. *Nature* 381, 667-673.
- Dudley,D.T., Pang,L., Decker,S.J., Bridges,A.J., and Saltiel,A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U. S. A* 92, 7686-7689.
- Dutil,E.M., Toker,A., and Newton,A.C. (1998). Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr. Biol.* 8, 1366-1375.
- Ehrlich,B.E., Kaftan,E., Bezprozvannaya,S., and Bezprozvanny,I. (1994). The pharmacology of intracellular Ca(2+)-release channels. *Trends Pharmacol. Sci.* 15, 145-149.
- El Sheikh,S.S., Domin,J., Tomtitchong,P., Abel,P., Stamp,G., and Lalani,E.N. (2003). Topographical expression of class IA and class II phosphoinositide 3-kinase enzymes in normal human tissues is consistent with a role in differentiation. *BMC. Clin. Pathol.* 3, 4.
- Elferink,J.G., Boonen,G.J., and de Koster,B.M. (1992). The role of calcium in neutrophil migration: the effect of calcium and calcium-antagonists in electroporated neutrophils. *Biochem. Biophys. Res. Commun.* 182, 864-869.
- Ellson,C.D., Andrews,S., Stephens,L.R., and Hawkins,P.T. (2002). The PX domain: a new phosphoinositide-binding module. *J. Cell Sci.* 115, 1099-1105.

- Embi,N., Rylatt,D.B., and Cohen,P. (1980). Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur. J. Biochem.* 107, 519-527.
- Empson,R.M. and Galione,A. (1997). Cyclic ADP-ribose enhances coupling between voltage-gated Ca²⁺ entry and intracellular Ca²⁺ release. *J. Biol. Chem.* 272, 20967-20970.
- Entschladen,F., Gunzer,M., Scheuffele,C.M., Niggemann,B., and Zanker,K.S. (2000). T lymphocytes and neutrophil granulocytes differ in regulatory signaling and migratory dynamics with regard to spontaneous locomotion and chemotaxis. *Cell Immunol.* 199, 104-114.
- Etienne-Manneville,S. and Hall,A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* 106, 489-498.
- Etienne-Manneville,S. and Hall,A. (2002). Rho GTPases in cell biology. *Nature* 420, 629-635.
- Fabbri,E., Spisani,S., Biondi,C., Barbin,L., Colamussi,M.L., Cariani,A., Traniello,S., Torrini,I., and Ferretti,M.E. (1997). Two for-Met-Leu-Phe-OMe analogues trigger selective neutrophil responses. A differential effect on cytosolic free Ca²⁺. *Biochim. Biophys. Acta* 1359, 233-240.
- Fang,X., Yu,S., Tanyi,J.L., Lu,Y., Woodgett,J.R., and Mills,G.B. (2002). Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. *Mol. Cell Biol.* 22, 2099-2110.
- Faure,M., Voyno-Yasenetskaya,T.A., and Bourne,H.R. (1994). cAMP and beta gamma subunits of heterotrimeric G proteins stimulate the mitogen-activated protein kinase pathway in COS-7 cells. *J. Biol. Chem.* 269, 7851-7854.
- Faux,M.C., Rollins,E.N., Edwards,A.S., Langeberg,L.K., Newton,A.C., and Scott,J.D. (1999). Mechanism of A-kinase-anchoring protein 79 (AKAP79) and protein kinase C interaction. *Biochem. J.* 343 Pt 2, 443-452.
- Faux,M.C. and Scott,J.D. (1997). Regulation of the AKAP79-protein kinase C interaction by Ca²⁺/Calmodulin. *J. Biol. Chem.* 272, 17038-17044.
- Feng,J., Ito,M., Ichikawa,K., Isaka,N., Nishikawa,M., Hartshorne,D.J., and Nakano,T. (1999). Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J. Biol. Chem.* 274, 37385-37390.
- Feng,Y., Broder,C.C., Kennedy,P.E., and Berger,E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272, 872-877.
- Fernandis,A.Z., Prasad,A., Band,H., Klosel,R., and Ganju,R.K. (2004). Regulation of CXCR4-mediated chemotaxis and chemoinvasion of breast cancer cells. *Oncogene* 23, 157-167.
- Fine,J.S., Byrnes,H.D., Zavodny,P.J., and Hipkin,R.W. (2001). Evaluation of signal transduction pathways in chemoattractant-induced human monocyte chemotaxis. *Inflammation* 25, 61-67.
- Forster,R., Schubel,A., Breitfeld,D., Kremmer,E., Renner-Muller,I., Wolf,E., and Lipp,M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99, 23-33.
- Foster,F.M., Traer,C.J., Abraham,S.M., and Fry,M.J. (2003). The phosphoinositide (PI) 3-kinase family. *J. Cell Sci.* 116, 3037-3040.
- Fox,J.A., Ung,K., Tanlimco,S.G., and Jirik,F.R. (2002). Disruption of a single Pten allele augments the chemotactic response of B lymphocytes to stromal cell-derived factor-1. *J. Immunol.* 169, 49-54.

- Fra, A.M., Locati, M., Otero, K., Sironi, M., Signorelli, P., Massardi, M.L., Gobbi, M., Vecchi, A., Sozzani, S., and Mantovani, A. (2003). Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J. Immunol.* 170, 2279-2282.
- Frame, S. and Cohen, P. (2001). GSK3 takes centre stage more than 20 years after its discovery. *Biochem. J.* 359, 1-16.
- Frank, S.R., Hatfield, J.C., and Casanova, J.E. (1998). Remodeling of the actin cytoskeleton is coordinately regulated by protein kinase C and the ADP-ribosylation factor nucleotide exchange factor ARNO. *Mol. Biol. Cell* 9, 3133-3146.
- Franke, T.F., Kaplan, D.R., Cantley, L.C., and Toker, A. (1997). Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. *Science* 275, 665-668.
- Frech, M., Andjelkovic, M., Ingley, E., Reddy, K.K., Falck, J.R., and Hemmings, B.A. (1997). High affinity binding of inositol phosphates and phosphoinositides to the pleckstrin homology domain of RAC/protein kinase B and their influence on kinase activity. *J. Biol. Chem.* 272, 8474-8481.
- Freeburn, R.W., Wright, K.L., Burgess, S.J., Astoul, E., Cantrell, D.A., and Ward, S.G. (2002). Evidence that SHIP-1 contributes to phosphatidylinositol 3,4,5-trisphosphate metabolism in T lymphocytes and can regulate novel phosphoinositide 3-kinase effectors. *J. Immunol.* 169, 5441-5450.
- Friel, D.D. and Tsien, R.W. (1992). Phase-dependent contributions from Ca^{2+} entry and Ca^{2+} release to caffeine-induced $[Ca^{2+}]_i$ oscillations in bullfrog sympathetic neurons. *Neuron* 8, 1109-1125.
- Frodin, M. and Gammeltoft, S. (1999). Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol. Cell Endocrinol.* 151, 65-77.
- Fruman, D.A. and Cantley, L.C. (2002). Phosphoinositide 3-kinase in immunological systems. *Semin. Immunol.* 14, 7-18.
- Fruman, D.A., Cantley, L.C., and Carpenter, C.L. (1996). Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85 alpha gene. *Genomics* 37, 113-121.
- Fukata, M., Nakagawa, M., and Kaibuchi, K. (2003). Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr. Opin. Cell Biol.* 15, 590-597.
- Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* 109, 611-623.
- Galione, A. and Churchill, G.C. (2000). Cyclic ADP ribose as a calcium-mobilizing messenger. *Sci. STKE.* 2000, E1.
- Galli, G., Chantry, D., Annunziato, F., Romagnani, P., Cosmi, L., Lazzeri, E., Manetti, R., Maggi, E., Gray, P.W., and Romagnani, S. (2000). Macrophage-derived chemokine production by activated human T cells in vitro and in vivo: preferential association with the production of type 2 cytokines. *Eur. J. Immunol.* 30, 204-210.
- Gamberucci, A., Giurisato, E., Pizzo, P., Tassi, M., Giunti, R., McIntosh, D.P., and Benedetti, A. (2002). Diacylglycerol activates the influx of extracellular cations in T-lymphocytes independently of intracellular calcium-store depletion and possibly involving endogenous TRP6 gene products. *Biochem. J.* 364, 245-254.
- Ganju, R.K., Brubaker, S.A., Chernock, R.D., Avraham, S., and Groopman, J.E. (2000). Beta-chemokine receptor CCR5 signals through SHP1, SHP2, and Syk. *J. Biol. Chem.* 275, 17263-17268.
- Ganju, R.K., Brubaker, S.A., Meyer, J., Dutt, P., Yang, Y., Qin, S., Newman, W., and Groopman, J.E. (1998a). The alpha-chemokine, stromal cell-derived factor-1alpha, binds to the transmembrane G-protein-coupled CXCR-4 receptor and activates multiple signal transduction pathways. *J. Biol. Chem.* 273, 23169-23175.

- Ganju,R.K., Dutt,P., Wu,L., Newman,W., Avraham,H., Avraham,S., and Groopman,J.E. (1998b). Beta-chemokine receptor CCR5 signals via the novel tyrosine kinase RAFTK. *Blood* 91, 791-797.
- Gao,Z. and Metz,W.A. (2003). Unraveling the chemistry of chemokine receptor ligands. *Chem. Rev.* 103, 3733-3752.
- Garrington,T.P. and Johnson,G.L. (1999). Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr. Opin. Cell Biol.* 11, 211-218.
- Gasteier,J.E., Madrid,R., Krautkramer,E., Schroder,S., Muranyi,W., Benichou,S., and Fackler,O.T. (2003). Activation of the Rac-binding Partner FHOD1 Induces Actin Stress Fibers via a ROCK-dependent Mechanism. *J. Biol. Chem.* 278, 38902-38912.
- Gelman,I.H. (2003). Pyk 2 FAKs, any two FAKs. *Cell Biol. Int.* 27, 507-510.
- Genazzani,A.A. and Billington,R.A. (2002). NAADP: an atypical Ca²⁺-release messenger? *Trends Pharmacol. Sci.* 23, 165-167.
- Genazzani,A.A. and Thorn,P. (2002). Calcium signalling: calcium goes global. *Curr. Biol.* 12, R432-R433.
- Gerard,C. and Rollins,B.J. (2001). Chemokines and disease. *Nat. Immunol.* 2, 108-115.
- Ghia,P., Transidico,P., Veiga,J.P., Schaniel,C., Sallusto,F., Matsushima,K., Sallan,S.E., Rolink,A.G., Mantovani,A., Nadler,L.M., and Cardoso,A.A. (2001). Chemoattractants MDC and TARC are secreted by malignant B-cell precursors following CD40 ligation and support the migration of leukemia-specific T cells. *Blood* 98, 533-540.
- Ghosh,P.M., Bedolla,R., Thomas,C.A., and Kreisberg,J.I. (2004). Role of protein kinase C in arginine vasopressin-stimulated ERK and p70S6 kinase phosphorylation. *J. Cell Biochem.* 91, 1109-1129.
- Gilbert,S.H., Perry,K., and Fay,F.S. (1994). Mediation of chemoattractant-induced changes in [Ca²⁺]_i and cell shape, polarity, and locomotion by InsP₃, DAG, and protein kinase C in newt eosinophils. *J. Cell Biol.* 127, 489-503.
- Gillitzer,R. and Goebeler,M. (2001). Chemokines in cutaneous wound healing. *J. Leukoc. Biol.* 69, 513-521.
- Gillooly,D.J., Raiborg,C., and Stenmark,H. (2003). Phosphatidylinositol 3-phosphate is found in microdomains of early endosomes. *Histochem. Cell Biol.* 120, 445-453.
- Gliki,G., Wheeler-Jones,C., and Zachary,I. (2002). Vascular endothelial growth factor induces protein kinase C (PKC)-dependent Akt/PKB activation and phosphatidylinositol 3'-kinase-mediates PKC delta phosphorylation: role of PKC in angiogenesis. *Cell Biol. Int.* 26, 751-759.
- Godiska,R., Chantry,D., Raport,C.J., Sozzani,S., Allavena,P., Leviten,D., Mantovani,A., and Gray,P.W. (1997). Human macrophage-derived chemokine (MDC), a novel chemoattractant for monocytes, monocyte-derived dendritic cells, and natural killer cells. *J. Exp. Med.* 185, 1595-1604.
- Gonzalo,J.A., Pan,Y., Lloyd,C.M., Jia,G.Q., Yu,G., Dussault,B., Powers,C.A., Proudfoot,A.E., Coyle,A.J., Gearing,D., and Gutierrez-Ramos,J.C. (1999). Mouse monocyte-derived chemokine is involved in airway hyperreactivity and lung inflammation. *J. Immunol.* 163, 403-411.
- Grammer,T.C. and Blenis,J. (1996). The serine protease inhibitors, tosylphenylalanine chloromethyl ketone and tosyllysine chloromethyl ketone, potently inhibit pp70s6k activation. *J. Biol. Chem.* 271, 23650-23652.
- Green,D.R. and Schuler,M. (2000). T cell development: some cells get all the breaks. *Nat. Immunol.* 1, 15-17.
- Gschwendt,M., Muller,H.J., Kielbassa,K., Zang,R., Kittstein,W., Rincke,G., and Marks,F. (1994). Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Commun.* 199, 93-98.

- Gu,J., Tamura,M., Pankov,R., Danen,E.H., Takino,T., Matsumoto,K., and Yamada,K.M. (1999). Shc and FAK differentially regulate cell motility and directionality modulated by PTEN. *J. Cell Biol.* *146*, 389-403.
- Guinamard,R., Okigaki,M., Schlessinger,J., and Ravetch,J.V. (2000). Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol.* *1*, 31-36.
- Guinamard,R., Signoret,N., Ishiai,M., Marsh,M., Kurosaki,T., Ravetch,J.V., and Masamichi,I. (1999). B cell antigen receptor engagement inhibits stromal cell-derived factor (SDF)-1 α chemotaxis and promotes protein kinase C (PKC)-induced internalization of CXCR4. *J. Exp. Med.* *189*, 1461-1466.
- Gunn,M.D., Ngo,V.N., Ansel,K.M., Ekland,E.H., Cyster,J.G., and Williams,L.T. (1998a). A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1. *Nature* *391*, 799-803.
- Gunn,M.D., Tangemann,K., Tam,C., Cyster,J.G., Rosen,S.D., and Williams,L.T. (1998b). A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. *Proc. Natl. Acad. Sci. U. S. A* *95*, 258-263.
- Guse,A.H., da Silva,C.P., Emmrich,F., Ashamu,G.A., Potter,B.V., and Mayr,G.W. (1995). Characterization of cyclic adenosine diphosphate-ribose-induced Ca²⁺ release in T lymphocyte cell lines. *J. Immunol.* *155*, 3353-3359.
- Gutkind,J.S. (2000). Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. *Sci. STKE.* *2000*, RE1.
- Haddad,E., Zugaza,J.L., Louache,F., Debili,N., Crouin,C., Schwarz,K., Fischer,A., Vainchenker,W., and Bertoglio,J. (2001). The interaction between Cdc42 and WASP is required for SDF-1-induced T- lymphocyte chemotaxis. *Blood* *97*, 33-38.
- Hahn,K., DeBiasio,R., and Taylor,D.L. (1992). Patterns of elevated free calcium and calmodulin activation in living cells. *Nature* *359*, 736-738.
- Hannigan,M., Zhan,L., Li,Z., Ai,Y., Wu,D., and Huang,C.K. (2002). Neutrophils lacking phosphoinositide 3-kinase gamma show loss of directionality during N-formyl-Met-Leu-Phe-induced chemotaxis. *Proc. Natl. Acad. Sci. U. S. A* *99*, 3603-3608.
- Haribabu,B., Richardson,R.M., Fisher,I., Sozzani,S., Peiper,S.C., Horuk,R., Ali,H., and Snyderman,R. (1997). Regulation of human chemokine receptors CXCR4. Role of phosphorylation in desensitization and internalization. *J. Biol. Chem.* *272*, 28726-28731.
- Hartwig,J.H., Thelen,M., Rosen,A., Janmey,P.A., Nairn,A.C., and Aderem,A. (1992). MARCKS is an actin filament crosslinking protein regulated by protein kinase C and calcium-calmodulin. *Nature* *356*, 618-622.
- Harwood,A. and Braga,V.M. (2003). Cdc42 & GSK-3: signals at the crossroads. *Nat. Cell Biol.* *5*, 275-277.
- Harwood,A.J. (2001). Regulation of GSK-3: a cellular multiprocessor. *Cell* *105*, 821-824.
- Hashii,M., Minabe,Y., and Higashida,H. (2000). cADP-ribose potentiates cytosolic Ca²⁺ elevation and Ca²⁺ entry via L-type voltage-activated Ca²⁺ channels in NG108-15 neuronal cells. *Biochem. J.* *345 Pt 2*, 207-215.
- Hashimoto,S., Suzuki,T., Dong,H.Y., Nagai,S., Yamazaki,N., and Matsushima,K. (1999). Serial analysis of gene expression in human monocyte-derived dendritic cells. *Blood* *94*, 845-852.
- Hawes,B.E., Luttrell,L.M., van Biesen,T., and Lefkowitz,R.J. (1996). Phosphatidylinositol 3-kinase is an early intermediate in the G beta gamma-mediated mitogen-activated protein kinase signaling pathway. *J. Biol. Chem.* *271*, 12133-12136.
- Hayashi,A., Seki,N., Hattori,A., Kozuma,S., and Saito,T. (1999). PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. *Biochim. Biophys. Acta* *1450*, 99-106.

Henkel,T., Machleidt,T., Alkalay,I., Kronke,M., Ben Neriah,Y., and Baeuerle,P.A. (1993). Rapid proteolysis of I kappa B-alpha is necessary for activation of transcription factor NF-kappa B. *Nature* 365, 182-185.

Henschler,R., Piiper,A., Bistran,R., and Mobest,D. (2003). SDF-1alpha-induced intracellular calcium transient involves Rho GTPase signalling and is required for migration of hematopoietic progenitor cells. *Biochem. Biophys. Res. Commun.* 311, 1067-1071.

Hernandez-Lopez,C., Varas,A., Sacedon,R., Jimenez,E., Munoz,J.J., Zapata,A.G., and Vicente,A. (2002). Stromal cell-derived factor 1/CXCR4 signaling is critical for early human T-cell development. *Blood* 99, 546-554.

Herold,K.C. and Taylor,L. (2003). Treatment of Type 1 diabetes with anti-CD3 monoclonal antibody: induction of immune regulation? *Immunol. Res.* 28, 141-150.

Hideshima,T., Chauhan,D., Hayashi,T., Podar,K., Akiyama,M., Gupta,D., Richardson,P., Munshi,N., and Anderson,K.C. (2002). The biological sequelae of stromal cell-derived factor-1alpha in multiple myeloma. *Mol. Cancer Ther.* 1, 539-544.

Hinton,H.J., Alessi,D.R., and Cantrell,D.A. (2004). The serine kinase phosphoinositide-dependent kinase 1 (PKD1) regulates T cell development. *Nat. Immunol.* 5, 539-545.

Hirata,H., Arima,M., Cheng,G., Honda,K., Fukushima,F., Yoshida,N., Eda,F., and Fukuda,T. (2003). Production of TARC and MDC by naive T cells in asthmatic patients. *J. Clin. Immunol.* 23, 34-45.

Hirsch,E., Katanaev,V.L., Garlanda,C., Azzolino,O., Pirola,L., Silengo,L., Sozzani,S., Mantovani,A., Altruda,F., and Wymann,M.P. (2000). Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287, 1049-1053.

Hixenbaugh,E.A., Goeckeler,Z.M., Papaiya,N.N., Wysolmerski,R.B., Silverstein,S.C., and Huang,A.J. (1997). Stimulated neutrophils induce myosin light chain phosphorylation and isometric tension in endothelial cells. *Am. J. Physiol* 273, H981-H988.

Hofman,P., d'Andrea,L., Guzman,E., Selva,E., Le Negrate,G., Far,D.F., Lemichez,E., Boquet,P., and Rossi,B. (1999). Neutrophil F-actin and myosin but not microtubules functionally regulate transepithelial migration induced by interleukin 8 across a cultured intestinal epithelial monolayer. *Eur. Cytokine Netw.* 10, 227-236.

Hogan,P.G., Chen,L., Nardone,J., and Rao,A. (2003). Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* 17, 2205-2232.

Homey,B., Muller,A., and Zlotnik,A. (2002). Chemokines: agents for the immunotherapy of cancer? *Nat. Rev. Immunol.* 2, 175-184.

Homey,B., Wang,W., Soto,H., Buchanan,M.E., Wiesenborn,A., Catron,D., Muller,A., McClanahan,T.K., Dieu-Nosjean,M.C., Orozco,R., Ruzicka,T., Lehmann,P., Oldham,E., and Zlotnik,A. (2000). Cutting edge: the orphan chemokine receptor G protein-coupled receptor- 2 (GPR-2, CCR10) binds the skin-associated chemokine CCL27 (CTACK/ALP/ILC). *J. Immunol.* 164, 3465-3470.

Horcher,M., Rot,A., Aschauer,H., and Besemer,J. (1998). IL-8 derivatives with a reduced potential to form homodimers are fully active in vitro and in vivo. *Cytokine* 10, 1-12.

Horuk,R., Chitnis,C.E., Darbonne,W.C., Colby,T.J., Rybicki,A., Hadley,T.J., and Miller,L.H. (1993). A receptor for the malarial parasite *Plasmodium vivax*: the erythrocyte chemokine receptor. *Science* 261, 1182-1184.

Houshmand,P. and Zlotnik,A. (2003). Therapeutic applications in the chemokine superfamily. *Curr. Opin. Chem. Biol.* 7, 457-460.

- Hresko,R.C., Murata,H., and Mueckler,M. (2003). Phosphoinositide-dependent kinase-2 is a distinct protein kinase enriched in a novel cytoskeletal fraction associated with adipocyte plasma membranes. *J. Biol. Chem.* 278, 21615-21622.
- Hu,Y., Qiao,L., Wang,S., Rong,S.B., Meuillet,E.J., Berggren,M., Gallegos,A., Powis,G., and Kozikowski,A.P. (2000). 3-(Hydroxymethyl)-bearing phosphatidylinositol ether lipid analogues and carbonate surrogates block PI3-K, Akt, and cancer cell growth. *J. Med. Chem.* 43, 3045-3051.
- Huang,A.J., Manning,J.E., Bandak,T.M., Ratau,M.C., Hanser,K.R., and Silverstein,S.C. (1993). Endothelial cell cytosolic free calcium regulates neutrophil migration across monolayers of endothelial cells. *J. Cell Biol.* 120, 1371-1380.
- Huang,Y., Paxton,W.A., Wolinsky,S.M., Neumann,A.U., Zhang,L., He,T., Kang,S., Ceradini,D., Jin,Z., Yazdanbakhsh,K., Kunstman,K., Erickson,D., Dragon,E., Landau,N.R., Phair,J., Ho,D.D., and Koup,R.A. (1996). The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat. Med.* 2, 1240-1243.
- Hunzicker-Dunn,M., Gurevich,V.V., Casanova,J.E., and Mukherjee,S. (2002). ARF6: a newly appreciated player in G protein-coupled receptor desensitization. *FEBS Lett.* 521, 3-8.
- Hwang,J.H., Hwang,J.H., Chung,H.K., Kim,D.W., Hwang,E.S., Suh,J.M., Kim,H., You,K.H., Kwon,O.Y., Ro,H.K., Jo,D.Y., and Shong,M. (2003). CXC chemokine receptor 4 expression and function in human anaplastic thyroid cancer cells. *J. Clin. Endocrinol. Metab.* 88, 408-416.
- Iellem,A., Mariani,M., Lang,R., Recalde,H., Panina-Bordignon,P., Sinigaglia,F., and D'Ambrosio,D. (2001). Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J. Exp. Med.* 194, 847-853.
- Iijima,M. and Devreotes,P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. *Cell* 109, 599-610.
- Iijima,M., Huang,Y.E., Luo,H.R., Vazquez,F., and Devreotes,P.N. (2004). Novel mechanism of PTEN regulation by its PIP2 binding motif is critical for chemotaxis. *J. Biol. Chem.*
- Ikura,M., Osawa,M., and Ames,J.B. (2002). The role of calcium-binding proteins in the control of transcription: structure to function. *Bioessays* 24, 625-636.
- Imai,T., Baba,M., Nishimura,M., Kakizaki,M., Takagi,S., and Yoshie,O. (1997). The T cell-directed CC chemokine TARC is a highly specific biological ligand for CC chemokine receptor 4. *J. Biol. Chem.* 272, 15036-15042.
- Imai,T., Chantry,D., Raport,C.J., Wood,C.L., Nishimura,M., Godiska,R., Yoshie,O., and Gray,P.W. (1998). Macrophage-derived chemokine is a functional ligand for the CC chemokine receptor 4. *J. Biol. Chem.* 273, 1764-1768.
- Imai,T., Nagira,M., Takagi,S., Kakizaki,M., Nishimura,M., Wang,J., Gray,P.W., Matsushima,K., and Yoshie,O. (1999). Selective recruitment of CCR4-bearing Th2 cells toward antigen- presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. *Int. Immunol.* 11, 81-88.
- Imai,T., Yoshida,T., Baba,M., Nishimura,M., Kakizaki,M., and Yoshie,O. (1996). Molecular cloning of a novel T cell-directed CC chemokine expressed in thymus by signal sequence trap using Epstein-Barr virus vector. *J. Biol. Chem.* 271, 21514-21521.
- Inaoki,M., Sato,S., Shirasaki,F., Mukaida,N., and Takehara,K. (2003). The frequency of type 2 CD8+ T cells is increased in peripheral blood from patients with psoriasis vulgaris. *J. Clin. Immunol.* 23, 269-278.

- Inngjerdengen,M., Al Aoukaty,A., Damaj,B., and Maghazachi,A.A. (1999). Differential utilization of cyclic ADP-ribose pathway by chemokines to induce the mobilization of intracellular calcium in NK cells. *Biochem. Biophys. Res. Commun.* 262, 467-472.
- Inngjerdengen,M., Damaj,B., and Maghazachi,A.A. (2000). Human NK cells express CC chemokine receptors 4 and 8 and respond to thymus and activation-regulated chemokine, macrophage-derived chemokine, and I-309. *J. Immunol.* 164, 4048-4054.
- Inngjerdengen,M., Torgersen,K.M., and Maghazachi,A.A. (2002). Lck is required for stromal cell-derived factor 1 alpha (CXCL12)- induced lymphoid cell chemotaxis. *Blood* 99, 4318-4325.
- Isakoff,S.J., Cardozo,T., Andreev,J., Li,Z., Ferguson,K.M., Abagyan,R., Lemmon,M.A., Aronheim,A., and Skolnik,E.Y. (1998). Identification and analysis of PH domain-containing targets of phosphatidylinositol 3-kinase using a novel in vivo assay in yeast. *EMBO J.* 17, 5374-5387.
- Ishida,T., Utsunomiya,A., Iida,S., Inagaki,H., Takatsuka,Y., Kusumoto,S., Takeuchi,G., Shimizu,S., Ito,M., Komatsu,H., Wakita,A., Eimoto,T., Matsushima,K., and Ueda,R. (2003). Clinical significance of CCR4 expression in adult T-cell leukemia/lymphoma: its close association with skin involvement and unfavorable outcome. *Clin. Cancer Res.* 9, 3625-3634.
- Ishizaki,T., Maekawa,M., Fujisawa,K., Okawa,K., Iwamatsu,A., Fujita,A., Watanabe,N., Saito,Y., Kakizuka,A., Morii,N., and Narumiya,S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* 15, 1885-1893.
- Islam,M.S. (2002). The ryanodine receptor calcium channel of beta-cells: molecular regulation and physiological significance. *Diabetes* 51, 1299-1309.
- Itoh,T. and Takenawa,T. (2002). Phosphoinositide-binding domains: Functional units for temporal and spatial regulation of intracellular signalling. *Cell Signal.* 14, 733-743.
- Iwabu,A., Smith,K., Allen,F.D., Lauffenburger,D.A., and Wells,A. (2004). Epidermal growth factor induces fibroblast contractility and motility via a protein kinase C delta-dependent pathway. *J. Biol. Chem.* 279, 14551-14560.
- Jaken,S. and Parker,P.J. (2000). Protein kinase C binding partners. *Bioessays* 22, 245-254.
- James,S.R., Downes,C.P., Gigg,R., Grove,S.J., Holmes,A.B., and Alessi,D.R. (1996). Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem. J.* 315 (Pt 3), 709-713.
- Jensen,C.J., Buch,M.B., Krag,T.O., Hemmings,B.A., Gammeltoft,S., and Frodin,M. (1999). 90-kDa ribosomal S6 kinase is phosphorylated and activated by 3-phosphoinositide-dependent protein kinase-1. *J. Biol. Chem.* 274, 27168-27176.
- Jiang,H., Kuang,Y., Wu,Y., Xie,W., Simon,M.I., and Wu,D. (1997). Roles of phospholipase C beta2 in chemoattractant-elicited responses. *Proc. Natl. Acad. Sci. U. S. A* 94, 7971-7975.
- Jo,Y., Matsumoto,T., Yada,S., Fujisawa,K., Esaki,M., Onai,N., Matsushima,K., and Iida,M. (2003). CCR4 is an up-regulated chemokine receptor of peripheral blood memory CD4+ T cells in Crohn's disease. *Clin. Exp. Immunol.* 132, 332-338.
- Johannes,F.J., Prestle,J., Eis,S., Oberhagemann,P., and Pfizenmaier,K. (1994). PKC ϵ is a novel, atypical member of the protein kinase C family. *J. Biol. Chem.* 269, 6140-6148.
- Jones,D., O'Hara,C., Kraus,M.D., Perez-Atayde,A.R., Shahsafaei,A., Wu,L., and Dorfman,D.M. (2000). Expression pattern of T-cell-associated chemokine receptors and their chemokines correlates with specific subtypes of T-cell non-Hodgkin lymphoma. *Blood* 96, 685-690.

- Jones,G.E., Prigmore,E., Calvez,R., Hogan,C., Dunn,G.A., Hirsch,E., Wymann,M.P., and Ridley,A.J. (2003). Requirement for PI 3-kinase gamma in macrophage migration to MCP-1 and CSF-1. *Exp. Cell Res.* 290, 120-131.
- Jones,P.F., Jakubowicz,T., and Hemmings,B.A. (1991a). Molecular cloning of a second form of rac protein kinase. *Cell Regul.* 2, 1001-1009.
- Jones,P.F., Jakubowicz,T., Pitossi,F.J., Maurer,F., and Hemmings,B.A. (1991b). Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc. Natl. Acad. Sci. U. S. A* 88, 4171-4175.
- Jordan,N.J., Kolios,G., Abbot,S.E., Sinai,M.A., Thompson,D.A., Petraki,K., and Westwick,J. (1999). Expression of functional CXCR4 chemokine receptors on human colonic epithelial cells. *J. Clin. Invest* 104, 1061-1069.
- Jou,S.T., Carpino,N., Takahashi,Y., Piekorz,R., Chao,J.R., Carpino,N., Wang,D., and Ihle,J.N. (2002). Essential, nonredundant role for the phosphoinositide 3-kinase p110delta in signaling by the B-cell receptor complex. *Mol. Cell Biol.* 22, 8580-8591.
- Kansra,V., Groves,C., Gutierrez-Ramos,J.C., and Polakiewicz,R.D. (2001). Phosphatidylinositol 3-kinase-dependent extracellular calcium influx is essential for CX(3)CR1-mediated activation of the mitogen-activated protein kinase cascade. *J. Biol. Chem.* 276, 31831-31838.
- Kashiwazaki,M., Tanaka,T., Kanda,H., Ebisuno,Y., Izawa,D., Fukuma,N., Akimitsu,N., Sekimizu,K., Monden,M., and Miyasaka,M. (2003). A high endothelial venule-expressing promiscuous chemokine receptor DARC can bind inflammatory, but not lymphoid, chemokines and is dispensable for lymphocyte homing under physiological conditions. *Int. Immunol.* 15, 1219-1227.
- Katschke,K.J., Jr., Rottman,J.B., Ruth,J.H., Qin,S., Wu,L., LaRosa,G., Ponath,P., Park,C.C., Pope,R.M., and Koch,A.E. (2001). Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthritis Rheum.* 44, 1022-1032.
- Katso,R., Okkenhaug,K., Ahmadi,K., White,S., Timms,J., and Waterfield,M.D. (2001). Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* 17, 615-675.
- Kawanabe,Y., Hashimoto,N., and Masaki,T. (2002). Effects of phosphoinositide 3-kinase on the endothelin-1-induced activation of voltage-independent Ca(2+) channels and mitogenesis in Chinese hamster ovary cells stably expressing endothelin(a) receptor. *Mol. Pharmacol.* 62, 756-761.
- Kawanabe,Y., Hashimoto,N., and Masaki,T. (2003). Role of phosphoinositide 3-kinase in the nonselective cation channel activation by endothelin-1/endothelinB receptor. *Am. J. Physiol Cell Physiol* 284, C506-C510.
- Kawano,Y., Fukata,Y., Oshiro,N., Amano,M., Nakamura,T., Ito,M., Matsumura,F., Inagaki,M., and Kaibuchi,K. (1999). Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. *J. Cell Biol.* 147, 1023-1038.
- Kawasaki,S., Takizawa,H., Yoneyama,H., Nakayama,T., Fujisawa,R., Izumizaki,M., Imai,T., Yoshie,O., Homma,I., Yamamoto,K., and Matsushima,K. (2001). Intervention of thymus and activation-regulated chemokine attenuates the development of allergic airway inflammation and hyperresponsiveness in mice. *J. Immunol.* 166, 2055-2062.
- Kazanietz,M.G. (2002). Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol. Pharmacol.* 61, 759-767.
- Keranen,L.M., Dutil,E.M., and Newton,A.C. (1995). Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. *Curr. Biol.* 5, 1394-1403.

- Kerkhoff,E. and Rapp,U.R. (2001). The Ras-Raf relationship: an unfinished puzzle. *Adv. Enzyme Regul.* **41**, 261-267.
- Kim,C.H., Hangoc,G., Cooper,S., Helgason,C.D., Yew,S., Humphries,R.K., Krystal,G., and Broxmeyer,H.E. (1999a). Altered responsiveness to chemokines due to targeted disruption of SHIP. *J. Clin. Invest* **104**, 1751-1759.
- Kim,H., Lee,H.S., Chang,K.T., Ko,T.H., Baek,K.J., and Kwon,N.S. (1995). Chloromethyl ketones block induction of nitric oxide synthase in murine macrophages by preventing activation of nuclear factor-kappa B. *J. Immunol.* **154**, 4741-4748.
- Kim,I.S., Ryang,Y.S., Kim,Y.S., Jang,S.W., Sung,H.J., Lee,Y.H., Kim,J., Na,D.S., and Ko,J. (2003). Leukotactin-1-induced ERK activation is mediated via Gi/Go protein/PLC/PKC delta/Ras cascades in HOS cells. *Life Sci.* **73**, 447-459.
- Kim,K.S., Rajarathnam,K., Clark-Lewis,I., and Sykes,B.D. (1996). Structural characterization of a monomeric chemokine: monocyte chemoattractant protein-3. *FEBS Lett.* **395**, 277-282.
- Kim,S., Jin,J., and Kunapuli,S.P. (2004). Akt activation in platelets depends on Gi signaling pathways. *J. Biol. Chem.* **279**, 4186-4195.
- Kim,S.H., Cleary,M.M., Fox,H.S., Chantry,D., and Sarvetnick,N. (2002). CCR4-bearing T cells participate in autoimmune diabetes. *J. Clin. Invest* **110**, 1675-1686.
- Kim,Y.H., Park,T.J., Lee,Y.H., Baek,K.J., Suh,P.G., Ryu,S.H., and Kim,K.T. (1999b). Phospholipase C-delta1 is activated by capacitative calcium entry that follows phospholipase C-beta activation upon bradykinin stimulation. *J. Biol. Chem.* **274**, 26127-26134.
- Kimura,K., Ito,M., Amano,M., Chihara,K., Fukata,Y., Nakafuku,M., Yamamori,B., Feng,J., Nakano,T., Okawa,K., Iwamatsu,A., and Kaibuchi,K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245-248.
- Klauck,T.M., Faux,M.C., Labudda,K., Langeberg,L.K., Jaken,S., and Scott,J.D. (1996). Coordination of three signaling enzymes by AKAP79, a mammalian scaffold protein. *Science* **271**, 1589-1592.
- Klippel,A., Kavanaugh,W.M., Pot,D., and Williams,L.T. (1997). A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. *Mol. Cell Biol.* **17**, 338-344.
- Knall,C., Worthen,G.S., and Johnson,G.L. (1997). Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc. Natl. Acad. Sci. U. S. A* **94**, 3052-3057.
- Knall,C., Young,S., Nick,J.A., Buhl,A.M., Worthen,G.S., and Johnson,G.L. (1996). Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *J. Biol. Chem.* **271**, 2832-2838.
- Ko,J., Kim,I.S., Jang,S.W., Lee,Y.H., Shin,S.Y., Min,d.S., and Na,D.S. (2002). Leukotactin-1/CCL15-induced chemotaxis signaling through CCR1 in HOS cells. *FEBS Lett.* **515**, 159-164.
- Koch,A.E., Volin,M.V., Woods,J.M., Kunkel,S.L., Connors,M.A., Harlow,L.A., Woodruff,D.C., Burdick,M.D., and Strieter,R.M. (2001). Regulation of angiogenesis by the C-X-C chemokines interleukin-8 and epithelial neutrophil activating peptide 78 in the rheumatoid joint. *Arthritis Rheum.* **44**, 31-40.
- Koch,W.J., Hawes,B.E., Inglese,J., Luttrell,L.M., and Lefkowitz,R.J. (1994). Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. *J. Biol. Chem.* **269**, 6193-6197.

- Kodaki,T., Woscholski,R., Hallberg,B., Rodriguez-Viciania,P., Downward,J., and Parker,P.J. (1994). The activation of phosphatidylinositol 3-kinase by Ras. *Curr. Biol.* 4, 798-806.
- Kofler,K., Erdel,M., Utermann,G., and Baier,G. (2002). Molecular genetics and structural genomics of the human protein kinase C gene module. *Genome Biol.* 3, RESEARCH0014.
- Koga,S., Auerbach,M.B., Engeman,T.M., Novick,A.C., Toma,H., and Fairchild,R.L. (1999). T cell infiltration into class II MHC-disparate allografts and acute rejection is dependent on the IFN-gamma-induced chemokine Mig. *J. Immunol.* 163, 4878-4885.
- Koivisto,L., Alavian,K., Hakkinen,L., Pelech,S., McCulloch,C.A., and Larjava,H. (2003). Glycogen synthase kinase-3 regulates formation of long lamellipodia in human keratinocytes. *J. Cell Sci.* 116, 3749-3760.
- Koizumi,S., Lipp,P., Berridge,M.J., and Bootman,M.D. (1999). Regulation of ryanodine receptor opening by luminal Ca(2+) underlies quantal Ca(2+) release in PC12 cells. *J. Biol. Chem.* 274, 33327-33333.
- Konishi,H., Kuroda,S., and Kikkawa,U. (1994). The pleckstrin homology domain of RAC protein kinase associates with the regulatory domain of protein kinase C zeta. *Biochem. Biophys. Res. Commun.* 205, 1770-1775.
- Koyasu,S. (2003). The role of PI3K in immune cells. *Nat. Immunol.* 4, 313-319.
- Kremer,K.N., Humphreys,T.D., Kumar,A., Qian,N.X., and Hedin,K.E. (2003). Distinct role of ZAP-70 and Src homology 2 domain-containing leukocyte protein of 76 kDa in the prolonged activation of extracellular signal-regulated protein kinase by the stromal cell-derived factor-1 alpha/CXCL12 chemokine. *J. Immunol.* 171, 360-367.
- Kroner,C., Eybrechts,K., and Akkerman,J.W. (2000). Dual regulation of platelet protein kinase B. *J. Biol. Chem.* 275, 27790-27798.
- Krugmann,S., Hawkins,P.T., Pryer,N., and Braselmann,S. (1999). Characterizing the interactions between the two subunits of the p101/p110gamma phosphoinositide 3-kinase and their role in the activation of this enzyme by G beta gamma subunits. *J. Biol. Chem.* 274, 17152-17158.
- Kuang,Y., Wu,Y., Jiang,H., and Wu,D. (1996). Selective G protein coupling by C-C chemokine receptors. *J. Biol. Chem.* 271, 3975-3978.
- Kuijpers,T.W., Hakkert,B.C., Hart,M.H., and Roos,D. (1992). Neutrophil migration across monolayers of cytokine-prestimulated endothelial cells: a role for platelet-activating factor and IL-8. *J. Cell Biol.* 117, 565-572.
- Kunkel,E.J., Campbell,D.J., and Butcher,E.C. (2003). Chemokines in lymphocyte trafficking and intestinal immunity. *Microcirculation.* 10, 313-323.
- Kurashima,K., Fujimura,M., Myou,S., Kasahara,K., Tachibana,H., Amemiya,N., Ishiura,Y., Onai,N., Matsushima,K., and Nakao,S. (2001). Effects of oral steroids on blood CXCR3+ and CCR4+ T cells in patients with bronchial asthma. *Am. J. Respir. Crit Care Med.* 164, 754-758.
- Kurosu,H., Maehama,T., Okada,T., Yamamoto,T., Hoshino,S., Fukui,Y., Ui,M., Hazeki,O., and Katada,T. (1997). Heterodimeric phosphoinositide 3-kinase consisting of p85 and p110beta is synergistically activated by the betagamma subunits of G proteins and phosphotyrosyl peptide. *J. Biol. Chem.* 272, 24252-24256.
- Kuschert,G.S., Coulin,F., Power,C.A., Proudfoot,A.E., Hubbard,R.E., Hoogewerf,A.J., and Wells,T.N. (1999). Glycosaminoglycans interact selectively with chemokines and modulate receptor binding and cellular responses. *Biochemistry* 38, 12959-12968.
- Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.

- Laffafian,I. and Hallett,M.B. (1995). Does cytosolic free Ca²⁺ signal neutrophil chemotaxis in response to formylated chemotactic peptide? *J. Cell Sci.* *108 (Pt 10)*, 3199-3205.
- Laudanna,C., Mochly-Rosen,D., Liron,T., Constantin,G., and Butcher,E.C. (1998). Evidence of zeta protein kinase C involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. *J. Biol. Chem.* *273*, 30306-30315.
- Lauffenburger,D.A. and Horwitz,A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* *84*, 359-369.
- Lawlor,M.A. and Alessi,D.R. (2001). PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Sci.* *114*, 2903-2910.
- Le Good,J.A., Ziegler,W.H., Parekh,D.B., Alessi,D.R., Cohen,P., and Parker,P.J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* *281*, 2042-2045.
- Lee,H.C. (1997). Mechanisms of calcium signaling by cyclic ADP-ribose and NAADP. *Physiol Rev.* *77*, 1133-1164.
- Lee,Y., Gotoh,A., Kwon,H.J., You,M., Kohli,L., Mantel,C., Cooper,S., Hangoc,G., Miyazawa,K., Ohyashiki,K., and Broxmeyer,H.E. (2002). Enhancement of intracellular signaling associated with hematopoietic progenitor cell survival in response to SDF-1/CXCL12 in synergy with other cytokines. *Blood* *99*, 4307-4317.
- Lemmon,M.A. and Ferguson,K.M. (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. *Biochem. J.* *350 Pt 1*, 1-18.
- Lentzsch,S., Gries,M., Janz,M., Bargou,R., Dorken,B., and Mapara,M.Y. (2003). Macrophage inflammatory protein 1-alpha (MIP-1 alpha) triggers migration and signaling cascades mediating survival and proliferation in multiple myeloma (MM) cells. *Blood* *101*, 3568-3573.
- Leopoldt,D., Hanck,T., Exner,T., Maier,U., Wetzker,R., and Nurnberg,B. (1998). Gbetagamma stimulates phosphoinositide 3-kinase-gamma by direct interaction with two domains of the catalytic p110 subunit. *J. Biol. Chem.* *273*, 7024-7029.
- Leslie,N.R. and Downes,C.P. (2002). PTEN: The down side of PI 3-kinase signalling. *Cell Signal.* *14*, 285-295.
- Leslie,N.R., Gray,A., Pass,I., Orchiston,E.A., and Downes,C.P. (2000). Analysis of the cellular functions of PTEN using catalytic domain and C-terminal mutations: differential effects of C-terminal deletion on signalling pathways downstream of phosphoinositide 3-kinase. *Biochem. J.* *346 Pt 3*, 827-833.
- Leung,T., Chen,X.Q., Manser,E., and Lim,L. (1996). The p160 RhoA-binding kinase ROK alpha is a member of a kinase family and is involved in the reorganization of the cytoskeleton. *Mol. Cell Biol.* *16*, 5313-5327.
- Leung,T., Manser,E., Tan,L., and Lim,L. (1995). A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J. Biol. Chem.* *270*, 29051-29054.
- Lewis,T.S., Shapiro,P.S., and Ahn,N.G. (1998). Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* *74*, 49-139.
- Lezcano-Meza,D., Negrete-Garcia,M.C., Dante-Escobedo,M., and Teran,L.M. (2003). The monocyte-derived chemokine is released in the bronchoalveolar lavage fluid of steady-state asthmatics. *Allergy* *58*, 1125-1130.
- Li,C., Wernig,F., Leitges,M., Hu,Y., and Xu,Q. (2003a). Mechanical stress-activated PKCdelta regulates smooth muscle cell migration. *FASEB J.* *17*, 2106-2108.

- Li,J., Dokka,S., Wang,L., Shi,X., Castranova,V., Yan,Y., Costa,M., and Huang,C. (2004a). Activation of aPKC is required for vanadate-induced phosphorylation of protein kinase B (Akt), but not p70S6k in mouse epidermal JB6 cells. *Mol. Cell Biochem.* 255, 217-225.
- Li,J., Yen,C., Liaw,D., Podsypanina,K., Bose,S., Wang,S.I., Puc,J., Miliareisis,C., Rodgers,L., McCombie,R., Bigner,S.H., Giovannella,B.C., Ittmann,M., Tycko,B., Hibshoosh,H., Wigler,M.H., and Parsons,R. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 275, 1943-1947.
- Li,S.W., Westwick,J., and Poli,C.T. (2002). Receptor-operated Ca^{2+} influx channels in leukocytes: a therapeutic target? *Trends Pharmacol. Sci.* 23, 63-70.
- Li,Y., Corradetti,M.N., Inoki,K., and Guan,K.L. (2004b). TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* 29, 32-38.
- Li,Z., Hannigan,M., Mo,Z., Liu,B., Lu,W., Wu,Y., Smrcka,A.V., Wu,G., Li,L., Liu,M., Huang,C.K., and Wu,D. (2003b). Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* 114, 215-227.
- Li,Z., Jiang,H., Xie,W., Zhang,Z., Smrcka,A.V., and Wu,D. (2000). Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* 287, 1046-1049.
- Liao,F., Ali,J., Greene,T., and Muller,W.A. (1997). Soluble domain 1 of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. *J. Exp. Med.* 185, 1349-1357.
- Liao,F., Huynh,H.K., Eiroa,A., Greene,T., Polizzi,E., and Muller,W.A. (1995). Migration of monocytes across endothelium and passage through extracellular matrix involve separate molecular domains of PECAM-1. *J. Exp. Med.* 182, 1337-1343.
- Lint,J.V., Rykx,A., Vantus,T., and Vandenheede,J.R. (2002). Getting to know protein kinase D. *Int. J. Biochem. Cell Biol.* 34, 577-581.
- Liscovitch,M., Czarny,M., Fiucci,G., Lavie,Y., and Tang,X. (1999). Localization and possible functions of phospholipase D isozymes. *Biochim. Biophys. Acta* 1439, 245-263.
- Liu,Q. and Dumont,D.J. (1997). Molecular cloning and chromosomal localization in human and mouse of the SH2-containing inositol phosphatase, INPP5D (SHIP). Amgen EST Program. *Genomics* 39, 109-112.
- Liu,R., Paxton,W.A., Choe,S., Ceradini,D., Martin,S.R., Horuk,R., MacDonald,M.E., Stuhlmann,H., Koup,R.A., and Landau,N.R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* 86, 367-377.
- Liu,W.S. and Heckman,C.A. (1998). The sevenfold way of PKC regulation. *Cell Signal.* 10, 529-542.
- Lloyd,C.M., Delaney,T., Nguyen,T., Tian,J., Martinez,A., Coyle,A.J., and Gutierrez-Ramos,J.C. (2000). CC chemokine receptor (CCR)3/eotaxin is followed by CCR4/monocyte- derived chemokine in mediating pulmonary T helper lymphocyte type 2 recruitment after serial antigen challenge in vivo. *J. Exp. Med.* 191, 265-274.
- Loetscher,P., Pellegrino,A., Gong,J.H., Mattioli,I., Loetscher,M., Bardi,G., Baggiolini,M., and Clark-Lewis,I. (2001). The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP10, are natural antagonists for CCR3. *J. Biol. Chem.* 276, 2986-2991.
- Lopez-Ilasaca,M., Crespo,P., Pellici,P.G., Gutkind,J.S., and Wetzker,R. (1997). Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science* 275, 394-397.

- Lorenzo, P.S., Beheshti, M., Pettit, G.R., Stone, J.C., and Blumberg, P.M. (2000). The guanine nucleotide exchange factor RasGRP is a high -affinity target for diacylglycerol and phorbol esters. *Mol. Pharmacol.* 57, 840-846.
- Lorenzo, P.S., Kung, J.W., Bottorff, D.A., Garfield, S.H., Stone, J.C., and Blumberg, P.M. (2001). Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res.* 61, 943-949.
- Lowman, H.B., Fairbrother, W.J., Slagle, P.H., Kabakoff, R., Liu, J., Shire, S., and Hebert, C.A. (1997). Monomeric variants of IL-8: effects of side chain substitutions and solution conditions upon dimer formation. *Protein Sci.* 6, 598-608.
- Lucas, A.D. and Greaves, D.R. (2001). Atherosclerosis: role of chemokines and macrophages. *Expert Reviews in Molecular Medicine* <http://www-ermm.cbcu.cam.ac.uk/01003696h.htm>.
- Lukyanenko, V., Gyorke, I., Wiesner, T.F., and Gyorke, S. (2001). Potentiation of Ca(2+) release by cADP-ribose in the heart is mediated by enhanced SR Ca(2+) uptake into the sarcoplasmic reticulum. *Circ. Res.* 89, 614-622.
- Luo, C., Burgeon, E., Carew, J.A., McCaffrey, P.G., Badalian, T.M., Lane, W.S., Hogan, P.G., and Rao, A. (1996). Recombinant NFAT1 (NFATp) is regulated by calcineurin in T cells and mediates transcription of several cytokine genes. *Mol. Cell Biol.* 16, 3955-3966.
- Luster, A.D. (2001). Antichemokine immunotherapy for allergic diseases. *Curr. Opin. Allergy Clin. Immunol.* 1, 561-567.
- Luster, A.D. (2002). The role of chemokines in linking innate and adaptive immunity. *Curr. Opin. Immunol.* 14, 129-135.
- Luther, S.A. and Cyster, J.G. (2001). Chemokines as regulators of T cell differentiation. *Nat. Immunol.* 2, 102-107.
- Luttrell, L.M., Hawes, B.E., van Biesen, T., Luttrell, D.K., Lansing, T.J., and Lefkowitz, R.J. (1996). Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. *J. Biol. Chem.* 271, 19443-19450.
- Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T., and Springer, T.A. (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in C. *Proc. Natl. Acad. Sci. U. S. A* 95, 9448-9453.
- Ma, Y.C., Huang, J., Ali, S., Lowry, W., and Huang, X.Y. (2000). Src tyrosine kinase is a novel direct effector of G proteins. *Cell* 102, 635-646.
- Macia, E., Chabre, M., and Franco, M. (2001). Specificities for the small G proteins ARF1 and ARF6 of the guanine nucleotide exchange factors ARNO and EFA6. *J. Biol. Chem.* 276, 24925-24930.
- Mackay, C.R. (1999). Dual personality of memory T cells. *Nature* 401, 659-660.
- Mackay, C.R. (2000). Follicular homing T helper (Th) cells and the Th1/Th2 paradigm. *J. Exp. Med.* 192, F31-F34.
- Mackay, C.R. (2001). Chemokines: immunology's high impact factors. *Nat. Immunol.* 2, 95-101.
- Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K., and Narumiya, S. (1999). Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science* 285, 895-898.
- Maffucci, T. and Falasca, M. (2001). Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. *FEBS Lett.* 506, 173-179.

- Maghazachi,A.A. (1997). Role of the heterotrimeric G proteins in stromal-derived factor-1alpha- induced natural killer cell chemotaxis and calcium mobilization. *Biochem. Biophys. Res. Commun.* 236, 270-274.
- Maghazachi,A.A. (2000). Intracellular signaling events at the leading edge of migrating cells. *Int. J. Biochem. Cell Biol.* 32, 931-943.
- Mahimainathan,L. and Ghosh,C.G. (2004). Inactivation of platelet-derived growth factor receptor by the tumor suppressor PTEN provides a novel mechanism of action of the phosphatase. *J. Biol. Chem.*
- Majka,M., Janowska-Wieczorek,A., Ratajczak,J., Kowalska,M.A., Vilaire,G., Pan,Z.K., Honczarenko,M., Marquez,L.A., Poncz,M., and Ratajczak,M.Z. (2000a). Stromal-derived factor 1 and thrombopoietin regulate distinct aspects of human megakaryopoiesis. *Blood* 96, 4142-4151.
- Majka,M., Ratajczak,J., Kowalska,M.A., and Ratajczak,M.Z. (2000b). Binding of stromal derived factor-1alpha (SDF-1alpha) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur. J. Haematol.* 64, 164-172.
- Mandeville,J.T. and Maxfield,F.R. (1997). Effects of buffering intracellular free calcium on neutrophil migration through three-dimensional matrices. *J. Cell Physiol* 171, 168-178.
- Manes,S., Mira,E., Gomez-Mouton,C., Zhao,Z.J., Lacalle,R.A., and Martinez,A. (1999). Concerted activity of tyrosine phosphatase SHP-2 and focal adhesion kinase in regulation of cell motility. *Mol. Cell Biol.* 19, 3125-3135.
- Mantovani,A. (1999). The chemokine system: redundancy for robust outputs. *Immunol. Today* 20, 254-257.
- Mantovani,A., Gray,P.A., Van Damme,J., and Sozzani,S. (2000). Macrophage-derived chemokine (MDC). *J. Leukoc. Biol.* 68, 400-404.
- Mao,M., Fang,X., Lu,Y., LaPushin,R., Bast,R.C., Jr., and Mills,G.B. (2000). Inhibition of growth-factor-induced phosphorylation and activation of protein kinase B/Akt by atypical protein kinase C in breast cancer cells. *Biochem. J.* 352 Pt 2, 475-482.
- March,M.E. and Ravichandran,K. (2002). Regulation of the immune response by SHIP. *Semin. Immunol.* 14, 37-47.
- Marinissen,M.J. and Gutkind,J.S. (2001). G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol. Sci.* 22, 368-376.
- Martinson,J.J., Chapman,N.H., Rees,D.C., Liu,Y.T., and Clegg,J.B. (1997). Global distribution of the CCR5 gene 32-basepair deletion. *Nat. Genet.* 16, 100-103.
- Martiny-Baron,G., Kazanietz,M.G., Mischak,H., Blumberg,P.M., Kochs,G., Hug,H., Marme,D., and Schachtele,C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J. Biol. Chem.* 268, 9194-9197.
- Maruyama,T., Kanaji,T., Nakade,S., Kanno,T., and Mikoshiba,K. (1997). 2APB, 2-aminoethoxydiphenyl borate, a membrane-penetrable modulator of Ins(1,4,5)P3-induced Ca2+ release. *J. Biochem. (Tokyo)* 122, 498-505.
- Matloubian,M., David,A., Engel,S., Ryan,J.E., and Cyster,J.G. (2000). A transmembrane CXC chemokine is a ligand for HIV-coreceptor Bonzo. *Nat. Immunol.* 1, 298-304.
- Matozaki,T., Nakanishi,H., and Takai,Y. (2000). Small G-protein networks: their crosstalk and signal cascades. *Cell Signal.* 12, 515-524.

- Matsui,S., Adachi,R., Kusui,K., Yamaguchi,T., Kasahara,T., Hayakawa,T., and Suzuki,K. (2001). U73122 inhibits the dephosphorylation and translocation of cofilin in activated macrophage-like U937 cells. *Cell Signal*. *13*, 17-22.
- Matsui,T., Amano,M., Yamamoto,T., Chihara,K., Nakafuku,M., Ito,M., Nakano,T., Okawa,K., Iwamatsu,A., and Kaibuchi,K. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. *EMBO J*. *15*, 2208-2216.
- Matsumoto,M., Ogawa,W., Hino,Y., Furukawa,K., Ono,Y., Takahashi,M., Ohba,M., Kuroki,T., and Kasuga,M. (2001). Inhibition of insulin-induced activation of Akt by a kinase-deficient mutant of the epsilon isozyyme of protein kinase C. *J. Biol. Chem*. *276*, 14400-14406.
- McConkey,M., Gillin,H., Webster,C.R., and Anwer,M.S. (2004). Cross-talk between Protein Kinases C{zeta} and B in Cyclic AMP-mediated Sodium Taurocholate Co-transporting Polypeptide Translocation in Hepatocytes. *J. Biol. Chem*. *279*, 20882-20888.
- McLeod,S.J., Li,A.H., Lee,R.L., Burgess,A.E., and Gold,M.R. (2002). The Rap GTPases regulate B cell migration toward the chemokine stromal cell-derived factor-1 (CXCL12): potential role for Rap2 in promoting B cell migration. *J. Immunol*. *169*, 1365-1371.
- McLeod,S.J., Shum,A.J., Lee,R.L., Takei,F., and Gold,M.R. (2004). The Rap GTPases regulate integrin-mediated adhesion, cell spreading, actin polymerization, and Pyk2 tyrosine phosphorylation in B lymphocytes. *J. Biol. Chem*. *279*, 12009-12019.
- Meili,R. and Firtel,R.A. (2003). Two poles and a compass. *Cell* *114*, 153-156.
- Mellado,M., Rodriguez-Frade,J.M., Aragay,A., del Real,G., Martin,A.M., Vila-Coro,A.J., Serrano,A., Mayor,F., Jr., and Martinez,A. (1998). The chemokine monocyte chemotactic protein 1 triggers Janus kinase 2 activation and tyrosine phosphorylation of the CCR2B receptor. *J. Immunol*. *161*, 805-813.
- Mellado,M., Rodriguez-Frade,J.M., Vila-Coro,A.J., Fernandez,S., Martin,d.A., Jones,D.R., Toran,J.L., and Martinez,A. (2001). Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *EMBO J*. *20*, 2497-2507.
- Mellor,H. and Parker,P.J. (1998). The extended protein kinase C superfamily. *Biochem. J*. *332* (Pt 2), 281-292.
- Meng,F., Liu,L., Chin,P.C., and D'Mello,S.R. (2002). Akt is a downstream target of NF-kappa B. *J. Biol. Chem*. *277*, 29674-29680.
- Merz,D., Liu,R., Johnson,K., and Terkeltaub,R. (2003). IL-8/CXCL8 and growth-related oncogene alpha/CXCL1 induce chondrocyte hypertrophic differentiation. *J. Immunol*. *171*, 4406-4415.
- Misawa,H., Ohtsubo,M., Copeland,N.G., Gilbert,D.J., Jenkins,N.A., and Yoshimura,A. (1998). Cloning and characterization of a novel class II phosphoinositide 3-kinase containing C2 domain. *Biochem. Biophys. Res. Commun*. *244*, 531-539.
- Mitchison,T.J. and Cramer,L.P. (1996). Actin-based cell motility and cell locomotion. *Cell* *84*, 371-379.
- Mitsui,G., Mitsui,K., Hirano,T., Ohara,O., Kato,M., and Niwano,Y. (2003). Kinetic profiles of sequential gene expressions for chemokines in mice with contact hypersensitivity. *Immunol. Lett*. *86*, 191-197.
- Mochly-Rosen,D. and Kauvar,L.M. (2000). Pharmacological regulation of network kinetics by protein kinase C localization. *Semin. Immunol*. *12*, 55-61.
- Mocsai,A., Zhang,H., Jakus,Z., Kitaura,J., Kawakami,T., and Lowell,C.A. (2003). G-protein-coupled receptor signaling in Syk-deficient neutrophils and mast cells. *Blood* *101*, 4155-4163.

- Moon,S.Y. and Zheng,Y. (2003). Rho GTPase-activating proteins in cell regulation. *Trends Cell Biol.* 13, 13-22.
- Moscat,J. and Diaz-Meco,M.T. (2000). The atypical protein kinase Cs. Functional specificity mediated by specific protein adapters. *EMBO Rep.* 1, 399-403.
- Moser,B. and Loetscher,P. (2001). Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2, 123-128.
- Mukai,H. (2003). The structure and function of PKN, a protein kinase having a catalytic domain homologous to that of PKC. *J. Biochem. (Tokyo)* 133, 17-27.
- Muller,W.A. (2003). Leukocyte-endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol.* 24, 327-334.
- Mullis,K., Faloona,F., Scharf,S., Saiki,R., Horn,G., and Erlich,H. (1986). Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harb. Symp. Quant. Biol.* 51 Pt 1, 263-273.
- Murphy,P.M., Baggiolini,M., Charo,I.F., Hebert,C.A., Horuk,R., Matsushima,K., Miller,L.H., Oppenheim,J.J., and Power,C.A. (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.* 52, 145-176.
- Myat,M.M., Anderson,S., Allen,L.A., and Aderem,A. (1997). MARCKS regulates membrane ruffling and cell spreading. *Curr. Biol.* 7, 611-614.
- Myers,M.P., Stolarov,J.P., Eng,C., Li,J., Wang,S.I., Wigler,M.H., Parsons,R., and Tonks,N.K. (1997). P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. U. S. A* 94, 9052-9057.
- Naga Prasad,S.V., Laporte,S.A., Chamberlain,D., Caron,M.G., Barak,L., and Rockman,H.A. (2002). Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. *J. Cell Biol.* 158, 563-575.
- Nagase,H., Kudo,K., Izumi,S., Ohta,K., Kobayashi,N., Yamaguchi,M., Matsushima,K., Morita,Y., Yamamoto,K., and Hirai,K. (2001). Chemokine receptor expression profile of eosinophils at inflamed tissue sites: Decreased CCR3 and increased CXCR4 expression by lung eosinophils. *J. Allergy Clin. Immunol.* 108, 563-569.
- Nakagawa,O., Fujisawa,K., Ishizaki,T., Saito,Y., Nakao,K., and Narumiya,S. (1996). ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett.* 392, 189-193.
- Newton,A.C. (1995a). Protein kinase C. Seeing two domains. *Curr. Biol.* 5, 973-976.
- Newton,A.C. (1995b). Protein kinase C: structure, function, and regulation. *J. Biol. Chem.* 270, 28495-28498.
- Newton,A.C. (2003). Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. *Biochem. J.* 370, 361-371.
- Ni,C.W., Wang,D.L., Lien,S.C., Cheng,J.J., Chao,Y.J., and Hsieh,H.J. (2003). Activation of PKC-epsilon and ERK1/2 participates in shear-induced endothelial MCP-1 expression that is repressed by nitric oxide. *J. Cell Physiol* 195, 428-434.
- Nibbs,R.J., Wylie,S.M., Yang,J., Landau,N.R., and Graham,G.J. (1997). Cloning and characterization of a novel promiscuous human beta- chemokine receptor D6. *J. Biol. Chem.* 272, 32078-32083.
- Nicholson,K.M. and Anderson,N.G. (2002). The protein kinase B/Akt signalling pathway in human malignancy. *Cell Signal.* 14, 381-395.

- Nishita,M., Aizawa,H., and Mizuno,K. (2002). Stromal cell-derived factor 1alpha activates LIM kinase 1 and induces cofilin phosphorylation for T-cell chemotaxis. *Mol. Cell Biol.* 22, 774-783.
- Niu,J., Profirovic,J., Pan,H., Vaiskunaite,R., and Voyno-Yasenetskaya,T. (2003). G Protein betagamma subunits stimulate p114RhoGEF, a guanine nucleotide exchange factor for RhoA and Rac1: regulation of cell shape and reactive oxygen species production. *Circ. Res.* 93, 848-856.
- Noguchi,N., Takasawa,S., Nata,K., Tohgo,A., Kato,I., Ikehata,F., Yonekura,H., and Okamoto,H. (1997). Cyclic ADP-ribose binds to FK506-binding protein 12.6 to release Ca²⁺ from islet microsomes. *J. Biol. Chem.* 272, 3133-3136.
- O'Neill,E. and Kolch,W. (2004). Conferring specificity on the ubiquitous Raf/MEK signalling pathway. *Br. J. Cancer* 90, 283-288.
- Oberlin,E., Amara,A., Bachelier,F., Bessia,C., Virelizier,J.L., Arenzana-Seisdedos,F., Schwartz,O., Heard,J.M., Clark-Lewis,I., Legler,D.F., Loetscher,M., Baggiolini,M., and Moser,B. (1996). The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* 382, 833-835.
- Ogilvie,P., Bardi,G., Clark-Lewis,I., Baggiolini,M., and Uguccioni,M. (2001). Eotaxin is a natural antagonist for CCR2 and an agonist for CCR5. *Blood* 97, 1920-1924.
- Ogilvie,P., Paoletti,S., Clark-Lewis,I., and Uguccioni,M. (2003). Eotaxin-3 is a natural antagonist for CCR2 and exerts a repulsive effect on human monocytes. *Blood*.
- Oh,E.S., Gu,H., Saxton,T.M., Timms,J.F., Hausdorff,S., Frevert,E.U., Kahn,B.B., Pawson,T., Neel,B.G., and Thomas,S.M. (1999). Regulation of early events in integrin signaling by protein tyrosine phosphatase SHP-2. *Mol. Cell Biol.* 19, 3205-3215.
- Oh,S.B., Endoh,T., Simen,A.A., Ren,D., and Miller,R.J. (2002). Regulation of calcium currents by chemokines and their receptors. *J. Neuroimmunol.* 123, 66-75.
- Okabe,S., Fukuda,S., and Broxmeyer,H.E. (2002). Src kinase, but not the src kinase family member p56lck, mediates stromal cell-derived factor 1alpha/CXCL12-induced chemotaxis of a T cell line. *J. Hematother. Stem Cell Res.* 11, 923-928.
- Okazaki,H., Kakurai,M., Hirata,D., Sato,H., Kamimura,T., Onai,N., Matsushima,K., Nakagawa,H., Kano,S., and Minota,S. (2002). Characterization of chemokine receptor expression and cytokine production in circulating CD4⁺ T cells from patients with atopic dermatitis: up-regulation of C-C chemokine receptor 4 in atopic dermatitis. *Clin. Exp. Allergy* 32, 1236-1242.
- Okigaki,M., Davis,C., Falasca,M., Harroch,S., Felsenfeld,D.P., Sheetz,M.P., and Schlessinger,J. (2003). Pyk2 regulates multiple signaling events crucial for macrophage morphology and migration. *Proc. Natl. Acad. Sci. U. S. A* 100, 10740-10745.
- Okkenhaug,K., Bilancio,A., Farjot,G., Priddle,H., Sancho,S., Peskett,E., Pearce,W., Meek,S.E., Salpekar,A., Waterfield,M.D., Smith,A.J., and Vanhaesebroeck,B. (2002). Impaired B and T cell antigen receptor signaling in p110delta PI 3-kinase mutant mice. *Science* 297, 1031-1034.
- Okkenhaug,K. and Vanhaesebroeck,B. (2003a). PI3K in lymphocyte development, differentiation and activation. *Nat. Rev. Immunol.* 3, 317-330.
- Okkenhaug,K. and Vanhaesebroeck,B. (2003b). PI3K-signalling in B- and T-cells: insights from gene-targeted mice. *Biochem. Soc. Trans.* 31, 270-274.
- Olson,T.S. and Ley,K. (2002). Chemokines and chemokine receptors in leukocyte trafficking. *Am. J. Physiol Regul. Integr. Comp Physiol* 283, R7-28.

- Ono,F., Nakagawa,T., Saito,S., Owada,Y., Sakagami,H., Goto,K., Suzuki,M., Matsuno,S., and Kondo,H. (1998). A novel class II phosphoinositide 3-kinase predominantly expressed in the liver and its enhanced expression during liver regeneration. *J. Biol. Chem.* 273, 7731-7736.
- Onuffer,J.J. and Horuk,R. (2002). Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends Pharmacol. Sci.* 23, 459-467.
- ORNSTEIN,L. (1964). DISC ELECTROPHORESIS. I. BACKGROUND AND THEORY. *Ann. N. Y. Acad. Sci.* 121, 321-349.
- Orr,J.W. and Newton,A.C. (1994). Intrapeptide regulation of protein kinase C. *J. Biol. Chem.* 269, 8383-8387.
- Owens,T., Wekerle,H., and Antel,J. (2001). Genetic models for CNS inflammation. *Nat. Med.* 7, 161-166.
- Page,K., Li,J., Zhou,L., Iasvovskaia,S., Corbit,K.C., Soh,J.W., Weinstein,I.B., Brasier,A.R., Lin,A., and Hershenson,M.B. (2003). Regulation of airway epithelial cell NF-kappa B-dependent gene expression by protein kinase C delta. *J. Immunol.* 170, 5681-5689.
- Panina-Bordignon,P., Papi,A., Mariani,M., Di Lucia,P., Casoni,G., Bellettato,C., Buonsanti,C., Miotto,D., Mapp,C., Villa,A., Arrigoni,G., Fabbri,L.M., and Sinigaglia,F. (2001). The C-C chemokine receptors CCR4 and CCR8 identify airway T cells of allergen-challenged atopic asthmatics. *J. Clin. Invest* 107, 1357-1364.
- Parekh,D.B., Ziegler,W., and Parker,P.J. (2000). Multiple pathways control protein kinase C phosphorylation. *EMBO J.* 19, 496-503.
- Paris,S., Beraud-Dufour,S., Robineau,S., Bigay,J., Antonny,B., Chabre,M., and Chardin,P. (1997). Role of protein-phospholipid interactions in the activation of ARF1 by the guanine nucleotide exchange factor Arno. *J. Biol. Chem.* 272, 22221-22226.
- Partida-Sanchez,S., Goodrich,S., Kusser,K., Oppenheimer,N., Randall,T.D., and Lund,F.E. (2004). Regulation of dendritic cell trafficking by the ADP-ribosyl cyclase CD38: impact on the development of humoral immunity. *Immunity.* 20, 279-291.
- Patel,S., Churchill,G.C., and Galione,A. (2001). Coordination of Ca²⁺ signalling by NAADP. *Trends Biochem. Sci.* 26, 482-489.
- Payrastre,B., Missy,K., Giuriato,S., Bodin,S., Plantavid,M., and Gratacap,M. (2001). Phosphoinositides: key players in cell signalling, in time and space. *Cell Signal.* 13, 377-387.
- Pendaries,C., Tronchere,H., Plantavid,M., and Payrastre,B. (2003). Phosphoinositide signaling disorders in human diseases. *FEBS Lett.* 546, 25-31.
- Pesesse,X., Deleu,S., De Smedt,F., Drayer,L., and Erneux,C. (1997). Identification of a second SH2-domain-containing protein closely related to the phosphatidylinositol polyphosphate 5-phosphatase SHIP. *Biochem. Biophys. Res. Commun.* 239, 697-700.
- Pettit,E.J. and Fay,F.S. (1998). Cytosolic free calcium and the cytoskeleton in the control of leukocyte chemotaxis. *Physiol Rev.* 78, 949-967.
- Philip,F., Guo,Y., and Scarlata,S. (2002). Multiple roles of pleckstrin homology domains in phospholipase Cbeta function. *FEBS Lett.* 531, 28-32.
- Platanias,L.C. (2003). Map kinase signaling pathways and hematologic malignancies. *Blood* 101, 4667-4679.
- Plater-Zyberk,C., Hoogewerf,A.J., Proudfoot,A.E., Power,C.A., and Wells,T.N. (1997). Effect of a CC chemokine receptor antagonist on collagen induced arthritis in DBA/1 mice. *Immunol. Lett.* 57, 117-120.

- Pogo,A.O. and Chaudhuri,A. (2000). The Duffy protein: a malarial and chemokine receptor. *Semin. Hematol.* 37, 122-129.
- Pollard,T.D. and Borisy,G.G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* 112, 453-465.
- Pollok-Kopp,B., Schwarze,K., Baradari,V.K., and Oppermann,M. (2003). Analysis of ligand-stimulated CC chemokine receptor 5 (CCR5) phosphorylation in intact cells using phosphosite-specific antibodies. *J. Biol. Chem.* 278, 2190-2198.
- Power,C.A. (2003). Knock out models to dissect chemokine receptor function in vivo. *J. Immunol. Methods* 273, 73-82.
- Power,C.A., Clemetson,J.M., Clemetson,K.J., and Wells,T.N. (1995a). Chemokine and chemokine receptor mRNA expression in human platelets. *Cytokine* 7, 479-482.
- Power,C.A., Meyer,A., Nemeth,K., Bacon,K.B., Hoogewerf,A.J., Proudfoot,A.E., and Wells,T.N. (1995b). Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. *J. Biol. Chem.* 270, 19495-19500.
- Power,C.A. and Wells,T.N. (1996). Cloning and characterization of human chemokine receptors. *Trends Pharmacol. Sci.* 17, 209-213.
- Powner,D.J. and Wakelam,M.J. (2002). The regulation of phospholipase D by inositol phospholipids and small GTPases. *FEBS Lett.* 531, 62-64.
- Proudfoot,A.E. (2002). Chemokine receptors: multifaceted therapeutic targets. *Nat. Rev. Immunol.* 2, 106-115.
- Proudfoot,A.E., Power,C.A., Rommel,C., and Wells,T.N. (2003). Strategies for chemokine antagonists as therapeutics. *Semin. Immunol.* 15, 57-65.
- Ptasznik,A., Urbanowska,E., Chinta,S., Costa,M.A., Katz,B.A., Stanislaus,M.A., Demir,G., Linnekin,D., Pan,Z.K., and Gewirtz,A.M. (2002). Crosstalk between BCR/ABL oncoprotein and CXCR4 signaling through a Src family kinase in human leukemia cells. *J. Exp. Med.* 196, 667-678.
- Qian,B., Soyer,O.S., Neubig,R.R., and Goldstein,R.A. (2003). Depicting a protein's two faces: GPCR classification by phylogenetic tree-based HMMs. *FEBS Lett.* 554, 95-99.
- Radhakrishna,H., Al Awar,O., Khachikian,Z., and Donaldson,J.G. (1999). ARF6 requirement for Rac ruffling suggests a role for membrane trafficking in cortical actin rearrangements. *J. Cell Sci.* 112 (Pt 6), 855-866.
- Raftopoulou,M. and Hall,A. (2004). Cell migration: Rho GTPases lead the way. *Dev. Biol.* 265, 23-32.
- Rao,A., Luo,C., and Hogan,P.G. (1997). Transcription factors of the NFAT family: regulation and function. *Annu. Rev. Immunol.* 15, 707-747.
- Rebecchi,M.J. and Penttyala,S.N. (2000). Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiol Rev.* 80, 1291-1335.
- Reif,K. and Cyster,J. (2002). The CDM protein DOCK2 in lymphocyte migration. *Trends Cell Biol.* 12, 368-373.
- Rhee,S.G. and Bae,Y.S. (1997). Regulation of phosphoinositide-specific phospholipase C isozymes. *J. Biol. Chem.* 272, 15045-15048.
- Rickert,P., Weiner,O.D., Wang,F., Bourne,H.R., and Servant,G. (2000). Leukocytes navigate by compass: roles of PI3Kgamma and its lipid products. *Trends Cell Biol.* 10, 466-473.

- Riento,K. and Ridley,A.J. (2003). Rocks: multifunctional kinases in cell behaviour. *Nat. Rev. Mol. Cell Biol.* *4*, 446-456.
- Rincon,M. (2001). MAP-kinase signaling pathways in T cells. *Curr. Opin. Immunol.* *13*, 339-345.
- Rincon,M., Flavell,R.A., and Davis,R.J. (2001). Signal transduction by MAP kinases in T lymphocytes. *Oncogene* *20*, 2490-2497.
- Risinger,J.I., Hayes,A.K., Berchuck,A., and Barrett,J.C. (1997). PTEN/MMAC1 mutations in endometrial cancers. *Cancer Res.* *57*, 4736-4738.
- Rizzuto,R., Pozzan,T., and Carafoli,E. (2002). Ca²⁺ on the move: ways and means to translate a multifarious signal. *Trends Pharmacol. Sci.* *23*, 348-350.
- Rodriguez-Frade,J.M., Vila-Coro,A.J., De Ana,A.M., Albar,J.P., Martinez,A., and Mellado,M. (1999a). The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc. Natl. Acad. Sci. U. S. A* *96*, 3628-3633.
- Rodriguez-Frade,J.M., Vila-Coro,A.J., Martin,A., Nieto,M., Sanchez-Madrid,F., Proudfoot,A.E., Wells,T.N., Martinez,A., and Mellado,M. (1999b). Similarities and differences in RA. *J. Cell Biol.* *144*, 755-765.
- Rodriguez-Viciana,P., Warne,P.H., Dhand,R., Vanhaesebroeck,B., Gout,I., Fry,M.J., Waterfield,M.D., and Downward,J. (1994). Phosphatidylinositol-3-OH kinase as a direct target of Ras. *Nature* *370*, 527-532.
- Rollins,B.J. (1997). Chemokines. *Blood* *90*, 909-928.
- Ron,D. and Kazanietz,M.G. (1999). New insights into the regulation of protein kinase C and novel phorbol ester receptors. *FASEB J.* *13*, 1658-1676.
- Rossi,D. and Zlotnik,A. (2000). The biology of chemokines and their receptors. *Annu. Rev. Immunol.* *18*, 217-242.
- Rottman,J.B., Smith,T.L., Ganley,K.G., Kikuchi,T., and Krueger,J.G. (2001). Potential role of the chemokine receptors CXCR3, CCR4, and the integrin α E β 7 in the pathogenesis of psoriasis vulgaris. *Lab Invest* *81*, 335-347.
- Rouleau,P., Vandal,K., Ryckman,C., Poubelle,P.E., Boivin,A., Talbot,M., and Tessier,P.A. (2003). The calcium-binding protein S100A12 induces neutrophil adhesion, migration, and release from bone marrow in mouse at concentrations similar to those found in human inflammatory arthritis. *Clin. Immunol.* *107*, 46-54.
- Rozycka,M., Lu,Y.J., Brown,R.A., Lau,M.R., Shipley,J.M., and Fry,M.J. (1998). cDNA cloning of a third human C2-domain-containing class II phosphoinositide 3-kinase, PI3K-C2gamma, and chromosomal assignment of this gene (PIK3C2G) to 12p12. *Genomics* *54*, 569-574.
- Ruth,J.H., Rottman,J.B., Katschke,K.J., Jr., Qin,S., Wu,L., LaRosa,G., Ponath,P., Pope,R.M., and Koch,A.E. (2001). Selective lymphocyte chemokine receptor expression in the rheumatoid joint. *Arthritis Rheum.* *44*, 2750-2760.
- Ryx,A., De Kimpe,L., Mikhalap,S., Vantus,T., Seufferlein,T., Vandenheede,J.R., and Van Lint,J. (2003). Protein kinase D: a family affair. *FEBS Lett.* *546*, 81-86.
- Sadhu,C., Dick,K., Tino,W.T., and Staunton,D.E. (2003a). Selective role of PI3K delta in neutrophil inflammatory responses. *Biochem. Biophys. Res. Commun.* *308*, 764-769.
- Sadhu,C., Masinovsky,B., Dick,K., Sowell,C.G., and Staunton,D.E. (2003b). Essential role of phosphoinositide 3-kinase delta in neutrophil directional movement. *J. Immunol.* *170*, 2647-2654.
- Sahai,E. and Marshall,C.J. (2002). RHO-GTPases and cancer. *Nat. Rev. Cancer* *2*, 133-142.

- Saito,H., Minamiya,Y., Kitamura,M., Saito,S., Enomoto,K., Terada,K., and Ogawa,J. (1998). Endothelial myosin light chain kinase regulates neutrophil migration across human umbilical vein endothelial cell monolayer. *J. Immunol.* *161*, 1533-1540.
- Sallusto,F. and Lanzavecchia,A. (2000). Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol. Rev.* *177*, 134-140.
- Sallusto,F., Lenig,D., Forster,R., Lipp,M., and Lanzavecchia,A. (1999a). Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* *401*, 708-712.
- Sallusto,F., Lenig,D., Mackay,C.R., and Lanzavecchia,A. (1998). Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* *187*, 875-883.
- Sallusto,F., Palermo,B., Lenig,D., Miettinen,M., Matikainen,S., Julkunen,I., Forster,R., Burgstahler,R., Lipp,M., and Lanzavecchia,A. (1999b). Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur. J. Immunol.* *29*, 1617-1625.
- Samson,M., Libert,F., Doranz,B.J., Rucker,J., Liesnard,C., Farber,C.M., Saragosti,S., Lapoumeroulie,C., Cogniaux,J., Forceille,C., Muyldermans,G., Verhofstede,C., Burtonboy,G., Georges,M., Imai,T., Rana,S., Yi,Y., Smyth,R.J., Collman,R.G., Doms,R.W., Vassart,G., and Parmentier,M. (1996a). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* *382*, 722-725.
- Samson,M., Soularue,P., Vassart,G., and Parmentier,M. (1996b). The genes encoding the human CC-chemokine receptors CC-CCR1 to CC-CCR5 (CMKBR1-CMKBR5) are clustered in the p21.3-p24 region of chromosome 3. *Genomics* *36*, 522-526.
- Samstag,Y., Eibert,S.M., Klemke,M., and Wabnitz,G.H. (2003). Actin cytoskeletal dynamics in T lymphocyte activation and migration. *J. Leukoc. Biol.* *73*, 30-48.
- Sanchez-Madrid,F. and del Pozo,M.A. (1999). Leukocyte polarization in cell migration and immune interactions. *EMBO J.* *18*, 501-511.
- Sanchez-Sanchez,N., Riol-Blanco,L., De La,R.G., Puig-Kroger,A., Garcia-Bordas,J., Martin,D., Longo,N., Cuadrado,A., Cabanas,C., Corbi,A.L., Sanchez-Mateos,P., and Rodriguez-Fernandez,J.L. (2004). Chemokine Receptor CCR7 Induces Intracellular signaling that Inhibits Apoptosis of Mature Dendritic Cells. *Blood*.
- Santy,L.C. and Casanova,J.E. (2001). Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J. Cell Biol.* *154*, 599-610.
- Saraiva,L., Fresco,P., Pinto,E., and Goncalves,J. (2003). Isoform-selectivity of PKC inhibitors acting at the regulatory and catalytic domain of mammalian PKC-alpha, -betaI, -delta, -eta and -zeta. *J. Enzyme Inhib. Med. Chem.* *18*, 475-483.
- Sasaki,T., Irie-Sasaki,J., Jones,R.G., Oliveira-dos-Santos,A.J., Stanford,W.L., Bolon,B., Wakeham,A., Itie,A., Bouchard,D., Kozieradzki,I., Joza,N., Mak,T.W., Ohashi,P.S., Suzuki,A., and Penninger,J.M. (2000). Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* *287*, 1040-1046.
- Sasaki,T., Suzuki,A., Sasaki,J., and Penninger,J.M. (2002). Phosphoinositide 3-kinases in immunity: lessons from knockout mice. *J. Biochem. (Tokyo)* *131*, 495-501.
- Sawyer,C., Sturge,J., Bennett,D.C., O'Hare,M.J., Allen,W.E., Bain,J., Jones,G.E., and Vanhaesebroeck,B. (2003). Regulation of breast cancer cell chemotaxis by the phosphoinositide 3-kinase p110delta. *Cancer Res.* *63*, 1667-1675.
- Scandella,E., Men,Y., Legler,D.F., Gillessen,S., Prikler,L., Ludewig,B., and Groettrup,M. (2003). CCL19/CCL21-triggered signal transduction and migration of dendritic cells require prostaglandin E2. *Blood*.

- Schaeffer, H.J. and Weber, M.J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* 19, 2435-2444.
- Schall, T.J. and Bacon, K.B. (1994). Chemokines, leukocyte trafficking, and inflammation. *Curr. Opin. Immunol.* 6, 865-873.
- Scharf, S.J., Horn, G.T., and Erlich, H.A. (1986). Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* 233, 1076-1078.
- Scheerens, H., Hessel, E., Waal-Malefyt, R., Leach, M.W., and Rennick, D. (2001). Characterization of chemokines and chemokine receptors in two murine models of inflammatory bowel disease: IL-10^{-/-} mice and Rag-2^{-/-} mice reconstituted with CD4⁺CD45RB^{high} T cells. *Eur. J. Immunol.* 31, 1465-1474.
- Scheid, M.P., Marignani, P.A., and Woodgett, J.R. (2002). Multiple phosphoinositide 3-kinase-dependent steps in activation of protein kinase B. *Mol. Cell Biol.* 22, 6247-6260.
- Scheid, M.P. and Woodgett, J.R. (2001). PKB/AKT: functional insights from genetic models. *Nat. Rev. Mol. Cell Biol.* 2, 760-768.
- Schenkel, A.R., Mamdouh, Z., Chen, X., Liebman, R.M., and Muller, W.A. (2002). CD99 plays a major role in the migration of monocytes through endothelial junctions. *Nat. Immunol.* 3, 143-150.
- Schini-Kerth, V.B., Boese, M., Busse, R., Fisslthaler, B., and Mulsch, A. (1997). N-alpha-tosyl-L-lysine chloromethylketone prevents expression of iNOS in vascular smooth muscle by blocking activation of NF-kappa B. *Arterioscler. Thromb. Vasc. Biol.* 17, 672-679.
- Schondorf, M., Bidlingmaier, F., and von Ruecker, A.A. (1993). Protein kinase C regulates IL-8 and fMLP induced cytoplasmic Ca²⁺ increase in human granulocytes by receptor modulation measurements by flow cytometry. *Biochem. Biophys. Res. Commun.* 197, 549-555.
- Schorr, W., Swandulla, D., and Zeilhofer, H.U. (1999). Mechanisms of IL-8-induced Ca²⁺ signaling in human neutrophil granulocytes. *Eur. J. Immunol.* 29, 897-904.
- Schuh, J.M., Power, C.A., Proudfoot, A.E., Kunkel, S.L., Lukacs, N.W., and Hogaboam, C.M. (2002). Airway hyperresponsiveness, but not airway remodeling, is attenuated during chronic pulmonary allergic responses to *Aspergillus* in CCR4^{-/-} mice. *FASEB J.* 16, 1313-1315.
- Schwarz, M.K. and Wells, T.N. (2002). New therapeutics that modulate chemokine networks. *Nat. Rev. Drug Discov.* 1, 347-358.
- Sekiya, T., Miyamasu, M., Imanishi, M., Yamada, H., Nakajima, T., Yamaguchi, M., Fujisawa, T., Pawankar, R., Sano, Y., Ohta, K., Ishii, A., Morita, Y., Yamamoto, K., Matsushima, K., Yoshie, O., and Hirai, K. (2000). Inducible expression of a Th2-type CC chemokine thymus- and activation- regulated chemokine by human bronchial epithelial cells. *J. Immunol.* 165, 2205-2213.
- Seminario, M.C. and Wange, R.L. (2002). Signaling pathways of D3-phosphoinositide-binding kinases in T cells and their regulation by PTEN. *Semin. Immunol.* 14, 27-36.
- Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287, 1037-1040.
- Shan, X., Czar, M.J., Bunnell, S.C., Liu, P., Liu, Y., Schwartzberg, P.L., and Wange, R.L. (2000). Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol. Cell Biol.* 20, 6945-6957.
- Shyamala, V. and Khoja, H. (1998). Interleukin-8 receptors R1 and R2 activate mitogen-activated protein kinases and induce c-fos, independent of Ras and Raf-1 in Chinese hamster ovary cells. *Biochemistry* 37, 15918-15924.

- Signoret,N., Oldridge,J., Pelchen-Matthews,A., Klasse,P.J., Tran,T., Brass,L.F., Rosenkilde,M.M., Schwartz,T.W., Holmes,W., Dallas,W., Luther,M.A., Wells,T.N., Hoxie,J.A., and Marsh,M. (1997). Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J. Cell Biol.* *139*, 651-664.
- Simmons,G., Reeves,J.D., Hibbitts,S., Stine,J.T., Gray,P.W., Proudfoot,A.E., and Clapham,P.R. (2000). Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. *Immunol. Rev.* *177*, 112-126.
- Simonsen,A., Wurmser,A.E., Emr,S.D., and Stenmark,H. (2001). The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* *13*, 485-492.
- Sly,L.M., Rauh,M.J., Kalesnikoff,J., Buchse,T., and Krystal,G. (2003). SHIP, SHIP2, and PTEN activities are regulated in vivo by modulation of their protein levels: SHIP is up-regulated in macrophages and mast cells by lipopolysaccharide. *Exp. Hematol.* *31*, 1170-1181.
- Small,J.V., Stradal,T., Vignal,E., and Rottner,K. (2002). The lamellipodium: where motility begins. *Trends Cell Biol.* *12*, 112-120.
- Smit,M.J., Verdijk,P., van der Raaij-Helmer EM, Navis,M., Hensbergen,P.J., Leurs,R., and Tensen,C.P. (2003). CXCR3-mediated chemotaxis of human T cells is regulated by a Gi- and phospholipase C-dependent pathway and not via activation of MEK/p44/p42 MAPK nor Akt/PI-3 kinase. *Blood* *102*, 1959-1965.
- Smith,R.J., Justen,J.M., McNab,A.R., Rosenbloom,C.L., Steele,A.N., Detmers,P.A., Anderson,D.C., and Manning,A.M. (1996). U-73122: a potent inhibitor of human polymorphonuclear neutrophil adhesion on biological surfaces and adhesion-related effector functions. *J. Pharmacol. Exp. Ther.* *278*, 320-329.
- Sodhi,A. and Biswas,S.K. (2002). Monocyte chemoattractant protein-1-induced activation of p42/44 MAPK and c-Jun in murine peritoneal macrophages: a potential pathway for macrophage activation. *J. Interferon Cytokine Res.* *22*, 517-526.
- Sodhi,A. and Sethi,G. (2003). Involvement of MAP kinase signal transduction pathway in UVB-induced activation of macrophages in vitro. *Immunol. Lett.* *90*, 123-130.
- Soede,R.D., Zeelenberg,I.S., Wijnands,Y.M., Kamp,M., and Roos,E. (2001). Stromal cell-derived factor-1-induced LFA-1 activation during in vivo migration of T cell hybridoma cells requires Gq/11, RhoA, and myosin, as well as Gi and Cdc42. *J. Immunol.* *166*, 4293-4301.
- Song,J., Khachikian,Z., Radhakrishna,H., and Donaldson,J.G. (1998). Localization of endogenous ARF6 to sites of cortical actin rearrangement and involvement of ARF6 in cell spreading. *J. Cell Sci.* *111* (Pt 15), 2257-2267.
- Sonnenburg,E.D., Gao,T., and Newton,A.C. (2001). The phosphoinositide-dependent kinase, PDK-1, phosphorylates conventional protein kinase C isozymes by a mechanism that is independent of phosphoinositide 3-kinase. *J. Biol. Chem.* *276*, 45289-45297.
- Sordella,R., Jiang,W., Chen,G.C., Curto,M., and Settleman,J. (2003). Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis. *Cell* *113*, 147-158.
- Soriano,S.F., Serrano,A., Hernanz-Falcon,P., De Ana,A.M., Monterrubio,M., Martinez,A., Rodriguez-Frade,J.M., and Mellado,M. (2003). Chemokines integrate JAK/STAT and G-protein pathways during chemotaxis and calcium flux responses. *Eur. J. Immunol.* *33*, 1328-1333.
- Sotsios,Y. and Ward,S.G. (2000). Phosphoinositide 3-kinase: a key biochemical signal for cell migration in response to chemokines. *Immunol. Rev.* *177*, 217-235.
- Sotsios,Y., Whittaker,G.C., Westwick,J., and Ward,S.G. (1999). The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J. Immunol.* *163*, 5954-5963.

Stam,J.C., Michiels,F., Van Der Kammen,R.A., Moolenaar,W.H., and Collard,J.G. (1998). Invasion of T-lymphoma cells: cooperation between Rho family GTPases and lysophospholipid receptor signaling. *EMBO J.* *17*, 4066-4074.

Steck,P.A., Pershouse,M.A., Jasser,S.A., Yung,W.K., Lin,H., Ligon,A.H., Langford,L.A., Baumgard,M.L., Hattier,T., Davis,T., Frye,C., Hu,R., Swedlund,B., Teng,D.H., and Tavtigian,S.V. (1997). Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* *15*, 356-362.

Stephens,L., Anderson,K., Stokoe,D., Erdjument-Bromage,H., Painter,G.F., Holmes,A.B., Gaffney,P.R., Reese,C.B., McCormick,F., Tempst,P., Coadwell,J., and Hawkins,P.T. (1998). Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* *279*, 710-714.

Stephens,L., Eguinoa,A., Corey,S., Jackson,T., and Hawkins,P.T. (1993). Receptor stimulated accumulation of phosphatidylinositol (3,4,5)-trisphosphate by G-protein mediated pathways in human myeloid derived cells. *EMBO J.* *12*, 2265-2273.

Stephens,L., Ellison,C., and Hawkins,P. (2002). Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr. Opin. Cell Biol.* *14*, 203-213.

Stephens,L.R., Eguinoa,A., Erdjument-Bromage,H., Lui,M., Cooke,F., Coadwell,J., Smrcka,A.S., Thelen,M., Cadwallader,K., Tempst,P., and Hawkins,P.T. (1997). The G beta gamma sensitivity of a PI3K is dependent upon a tightly associated adaptor, p101. *Cell* *89*, 105-114.

Stokoe,D., Stephens,L.R., Copeland,T., Gaffney,P.R., Reese,C.B., Painter,G.F., Holmes,A.B., McCormick,F., and Hawkins,P.T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* *277*, 567-570.

Stork,P.J. (2003). Does Rap1 deserve a bad Rap? *Trends Biochem. Sci.* *28*, 267-275.

Stossel,T.P. (1993). On the crawling of animal cells. *Science* *260*, 1086-1094.

Straussman,R., Even,L., and Ravid,S. (2001). Myosin II heavy chain isoforms are phosphorylated in an EGF-dependent manner: involvement of protein kinase C. *J. Cell Sci.* *114*, 3047-3057.

Strieter,R.M., Polverini,P.J., Arenberg,D.A., and Kunkel,S.L. (1995a). The role of CXC chemokines as regulators of angiogenesis. *Shock* *4*, 155-160.

Strieter,R.M., Polverini,P.J., Kunkel,S.L., Arenberg,D.A., Burdick,M.D., Kasper,J., Dzuiba,J., Van Damme,J., Walz,A., Marriott,D., and . (1995b). The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* *270*, 27348-27357.

Strosberg,A.D. (1991). Structure/function relationship of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur. J. Biochem.* *196*, 1-10.

Struyf,S., Proost,P., Sozzani,S., Mantovani,A., Wuyts,A., De Clercq,E., Schols,D., and Van Damme,J. (1998). Enhanced anti-HIV-1 activity and altered chemotactic potency of NH₂-terminally processed macrophage-derived chemokine (MDC) imply an additional MDC receptor. *J. Immunol.* *161*, 2672-2675.

Struyf,S., Stoops,G., Van Coillie,E., Gouwy,M., Schutyser,E., Lenaerts,J.P., Fiten,P., Van,A., I, Proost,P., Opdenakker,G., and Van Damme,J. (2001). Gene cloning of a new plasma CC chemokine, activating and attracting myeloid cells in synergy with other chemoattractants. *Biochemistry* *40*, 11715-11722.

Sturany,S., Van Lint,J., Muller,F., Wilda,M., Hameister,H., Hocker,M., Brey,A., Gern,U., Vandenheede,J., Gress,T., Adler,G., and Seufferlein,T. (2001). Molecular cloning and characterization of the human protein kinase D2. A novel member of the protein kinase D family of serine threonine kinases. *J. Biol. Chem.* *276*, 3310-3318.

Suire,S., Hawkins,P., and Stephens,L. (2002). Activation of phosphoinositide 3-kinase gamma by Ras. *Curr. Biol.* *12*, 1068-1075.

Sullivan,S.K., McGrath,D.A., Grigoriadis,D., and Bacon,K.B. (1999a). Pharmacological and signaling analysis of human chemokine receptor CCR- 7 stably expressed in HEK-293 cells: high-affinity binding of recombinant ligands MIP-3beta and SLC stimulates multiple signaling cascades. *Biochem. Biophys. Res. Commun.* *263*, 685-690.

Sullivan,S.K., McGrath,D.A., Liao,F., Boehme,S.A., Farber,J.M., and Bacon,K.B. (1999b). MIP-3alpha induces human eosinophil migration and activation of the mitogen-activated protein kinases (p42/p44 MAPK). *J. Leukoc. Biol.* *66*, 674-682.

Suzuki,G., Sawa,H., Kobayashi,Y., Nakata,Y., Nakagawa,K., Uzawa,A., Sakiyama,H., Kakinuma,S., Iwabuchi,K., and Nagashima,K. (1999). Pertussis toxin-sensitive signal controls the trafficking of thymocytes across the corticomedullary junction in the thymus. *J. Immunol.* *162*, 5981-5985.

Suzuki,Y., Rahman,M., and Mitsuya,H. (2001). Diverse transcriptional response of CD4(+) T cells to stromal cell- derived factor (SDF)-1: cell survival promotion and priming effects of SDF-1 on CD4(+) T cells. *J. Immunol.* *167*, 3064-3073.

Swaminathan,G.J., Holloway,D.E., Colvin,R.A., Campanella,G.K., Papageorgiou,A.C., Luster,A.D., and Acharya,K.R. (2003). Crystal Structures of Oligomeric Forms of the IP-10/CXCL10 Chemokine. *Structure. (Camb.)* *11*, 521-532.

Tachibana,K., Hirota,S., Iizasa,H., Yoshida,H., Kawabata,K., Kataoka,Y., Kitamura,Y., Matsushima,K., Yoshida,N., Nishikawa,S., Kishimoto,T., and Nagasawa,T. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* *393*, 591-594.

Takai,Y., Kishimoto,A., Inoue,M., and Nishizuka,Y. (1977). Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. *J. Biol. Chem.* *252*, 7603-7609.

Tamura,M., Gu,J., Danen,E.H., Takino,T., Miyamoto,S., and Yamada,K.M. (1999). PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway. *J. Biol. Chem.* *274*, 20693-20703.

Tamura,M., Gu,J., Matsumoto,K., Aota,S., Parsons,R., and Yamada,K.M. (1998). Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN. *Science* *280*, 1614-1617.

Tanaka,Y., Adams,D.H., Hubscher,S., Hirano,H., Siebenlist,U., and Shaw,S. (1993). T-cell adhesion induced by proteoglycan-immobilized cytokine MIP-1 beta. *Nature* *361*, 79-82.

Tang,X., Batty,I.H., and Downes,C.P. (2002). Muscarinic receptors mediate phospholipase C-dependent activation of protein kinase B via Ca²⁺, ErbB3, and phosphoinositide 3-kinase in 1321N1 astrocytoma cells. *J. Biol. Chem.* *277*, 338-344.

Tashiro,H., Blazes,M.S., Wu,R., Cho,K.R., Bose,S., Wang,S.I., Li,J., Parsons,R., and Ellenson,L.H. (1997). Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. *Cancer Res.* *57*, 3935-3940.

Taylor,J.R., Jr., Kimbrell,K.C., Scoggins,R., Delaney,M., Wu,L., and Camerini,D. (2001). Expression and function of chemokine receptors on human thymocytes: implications for infection by human immunodeficiency virus type 1. *J. Virol.* *75*, 8752-8760.

Thelen,M. and Baggiolini,M. (2001). Is dimerization of chemokine receptors functionally relevant? *Sci. STKE.* *2001*, E34.

- Thompson,S.D., Luyrink,L.K., Graham,T.B., Tsoras,M., Ryan,M., Passo,M.H., and Glass,D.N. (2001). Chemokine receptor CCR4 on CD4+ T cells in juvenile rheumatoid arthritis synovial fluid defines a subset of cells with increased IL- 4:IFN-gamma mRNA ratios. *J. Immunol.* *166*, 6899-6906.
- Tilton,B., Andjelkovic,M., Didichenko,S.A., Hemmings,B.A., and Thelen,M. (1997). G-Protein-coupled receptors and Fcγ-receptors mediate activation of Akt/protein kinase B in human phagocytes. *J. Biol. Chem.* *272*, 28096-28101.
- Tilton,B., Ho,L., Oberlin,E., Loetscher,P., Baleux,F., Clark-Lewis,I., and Thelen,M. (2000). Signal transduction by CXC chemokine receptor 4. Stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal- regulated kinase 2 activation in T lymphocytes. *J. Exp. Med.* *192*, 313-324.
- Toda,K., Nogami,M., Murakami,K., Kanaho,Y., and Nakayama,K. (1999). Colocalization of phospholipase D1 and GTP-binding-defective mutant of ADP-ribosylation factor 6 to endosomes and lysosomes. *FEBS Lett.* *442*, 221-225.
- Toker,A. and Newton,A.C. (2000a). Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J. Biol. Chem.* *275*, 8271-8274.
- Toker,A. and Newton,A.C. (2000b). Cellular signaling: pivoting around PDK-1. *Cell* *103*, 185-188.
- Torres,J. and Pulido,R. (2001). The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. *J. Biol. Chem.* *276*, 993-998.
- Torres,J., Rodriguez,J., Myers,M.P., Valiente,M., Graves,J.D., Tonks,N.K., and Pulido,R. (2003). Phosphorylation-regulated cleavage of the tumor suppressor PTEN by caspase-3: implications for the control of protein stability and PTEN-protein interactions. *J. Biol. Chem.* *278*, 30652-30660.
- Totsukawa,G., Yamakita,Y., Yamashiro,S., Hartshorne,D.J., Sasaki,Y., and Matsumura,F. (2000). Distinct roles of ROCK (Rho-kinase) and MLCK in spatial regulation of MLC phosphorylation for assembly of stress fibers and focal adhesions in 3T3 fibroblasts. *J. Cell Biol.* *150*, 797-806.
- Tsujio,I., Tanaka,T., Kudo,T., Nishikawa,T., Shinozaki,K., Grundke-Iqbal,I., Iqbal,K., and Takeda,M. (2000). Inactivation of glycogen synthase kinase-3 by protein kinase C delta: implications for regulation of tau phosphorylation. *FEBS Lett.* *469*, 111-117.
- Turner,C.E. and Brown,M.C. (2001). Cell motility: ARNOand ARF6 at the cutting edge. *Curr. Biol.* *11*, R875-R877.
- Turner,L., Ward,S.G., and Westwick,J. (1995). RANTES-activated human T lymphocytes. A role for phosphoinositide 3- kinase. *J. Immunol.* *155*, 2437-2444.
- Turner,S.J., Domin,J., Waterfield,M.D., Ward,S.G., and Westwick,J. (1998). The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2α. *J. Biol. Chem.* *273*, 25987-25995.
- Uchida,D., Begum,N.M., Almofti,A., Nakashiro,K., Kawamata,H., Tateishi,Y., Hamakawa,H., Yoshida,H., and Sato,M. (2003). Possible role of stromal-cell-derived factor-1/CXCR4 signaling on lymph node metastasis of oral squamous cell carcinoma. *Exp. Cell Res.* *290*, 289-302.
- Uchida,T., Suto,H., Ra,C., Ogawa,H., Kobata,T., and Okumura,K. (2002). Preferential expression of T(h)2-type chemokine and its receptor in atopic dermatitis. *Int. Immunol.* *14*, 1431-1438.
- Uehara,S., Grinberg,A., Farber,J.M., and Love,P.E. (2002). A role for CCR9 in T lymphocyte development and migration. *J. Immunol.* *168*, 2811-2819.

- Ueki,K., Fruman,D.A., Yballe,C.M., Fasshauer,M., Klein,J., Asano,T., Cantley,L.C., and Kahn,C.R. (2003). Positive and negative roles of p85 alpha and p85 beta regulatory subunits of phosphoinositide 3-kinase in insulin signaling. *J. Biol. Chem.* 278, 48453-48466.
- Urso,B., Brown,R.A., O'Rahilly,S., Shepherd,P.R., and Siddle,K. (1999). The alpha-isoform of class II phosphoinositide 3-kinase is more effectively activated by insulin receptors than IGF receptors, and activation requires receptor NPEY motifs. *FEBS Lett.* 460, 423-426.
- Valverde,A.M., Sinnott-Smith,J., Van Lint,J., and Rozengurt,E. (1994). Molecular cloning and characterization of protein kinase D: a target for diacylglycerol and phorbol esters with a distinctive catalytic domain. *Proc. Natl. Acad. Sci. U. S. A* 91, 8572-8576.
- Van Aelst,L. and D'Souza-Schorey,C. (1997). Rho GTPases and signaling networks. *Genes Dev.* 11, 2295-2322.
- van den,B.A., Visser,L., and Poppema,S. (1999). High expression of the CC chemokine TARC in Reed-Sternberg cells. A possible explanation for the characteristic T-cell infiltrate in Hodgkin's lymphoma. *Am. J. Pathol.* 154, 1685-1691.
- Van Lint,J., Rykx,A., Maeda,Y., Vantus,T., Sturany,S., Malhotra,V., Vandenheede,J.R., and Seufferlein,T. (2002). Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol.* 12, 193-200.
- Vancurova,I., Miskolci,V., and Davidson,D. (2001). NF-kappa B activation in tumor necrosis factor alpha-stimulated neutrophils is mediated by protein kinase Cdelta. Correlation to nuclear Ikappa Balpha. *J. Biol. Chem.* 276, 19746-19752.
- Vanhaesebroeck,B. and Alessi,D.R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.* 346 Pt 3, 561-576.
- Vanhaesebroeck,B., Jones,G.E., Allen,W.E., Zicha,D., Hooshmand-Rad,R., Sawyer,C., Wells,C., Waterfield,M.D., and Ridley,A.J. (1999). Distinct PI(3)Ks mediate mitogenic signalling and cell migration in macrophages. *Nat. Cell Biol.* 1, 69-71.
- Vanhaesebroeck,B., Leever,S.J., Ahmadi,K., Timms,J., Katso,R., Driscoll,P.C., Woscholski,R., Parker,P.J., and Waterfield,M.D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* 70, 535-602.
- Vanhaesebroeck,B., Leever,S.J., Panayotou,G., and Waterfield,M.D. (1997a). Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci.* 22, 267-272.
- Vanhaesebroeck,B., Welham,M.J., Kotani,K., Stein,R., Warne,P.H., Zvelebil,M.J., Higashi,K., Volinia,S., Downward,J., and Waterfield,M.D. (1997b). P110delta, a novel phosphoinositide 3-kinase in leukocytes. *Proc. Natl. Acad. Sci. U. S. A* 94, 4330-4335.
- Vazquez,F., Ramaswamy,S., Nakamura,N., and Sellers,W.R. (2000). Phosphorylation of the PTEN tail regulates protein stability and function. *Mol. Cell Biol.* 20, 5010-5018.
- Velasco,G., Armstrong,C., Morrice,N., Frame,S., and Cohen,P. (2002). Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. *FEBS Lett.* 527, 101-104.
- Venkateswarlu,K., Oatey,P.B., Tavaré,J.M., and Cullen,P.J. (1998). Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. *Curr. Biol.* 8, 463-466.
- Vestergaard,C., Bang,K., Gesser,B., Yoneyama,H., Matsushima,K., and Larsen,C.G. (2000). A Th2 chemokine, TARC, produced by keratinocytes may recruit CLA+CCR4+ lymphocytes into lesional atopic dermatitis skin. *J. Invest. Dermatol.* 115, 640-646.

Vestergaard,C., Deleuran,M., Gesser,B., and Gronhoj,L.C. (2003). Expression of the T-helper 2-specific chemokine receptor CCR4 on CCR10-positive lymphocytes in atopic dermatitis skin but not in psoriasis skin. *Br. J. Dermatol.* *149*, 457-463.

Vestergaard,C., Yoneyama,H., Murai,M., Nakamura,K., Tamaki,K., Terashima,Y., Imai,T., Yoshie,O., Irimura,T., Mizutani,H., and Matsushima,K. (1999). Overproduction of Th2-specific chemokines in NC/Nga mice exhibiting atopic dermatitis-like lesions. *J. Clin. Invest* *104*, 1097-1105.

Vestweber,D. and Blanks,J.E. (1999). Mechanisms that regulate the function of the selectins and their ligands. *Physiol Rev.* *79*, 181-213.

Viard,P., Exner,T., Maier,U., Mironneau,J., Nurnberg,B., and Macrez,N. (1999). Gbetagamma dimers stimulate vascular L-type Ca²⁺ channels via phosphoinositide 3-kinase. *FASEB J.* *13*, 685-694.

Vicente-Manzanares,M., Cabrero,J.R., Rey,M., Perez-Martinez,M., Ursa,A., Itoh,K., and Sanchez-Madrid,F. (2002). A role for the Rho-p160 Rho coiled-coil kinase axis in the chemokine stromal cell-derived factor-1alpha-induced lymphocyte actomyosin and microtubular organization and chemotaxis. *J. Immunol.* *168*, 400-410.

Vicente-Manzanares,M., Rey,M., Jones,D.R., Sancho,D., Mellado,M., Rodriguez-Frade,J.M., del Pozo,M.A., Yanez-Mo,M., De Ana,A.M., Martinez,A., Merida,I., and Sanchez-Madrid,F. (1999). Involvement of phosphatidylinositol 3-kinase in stromal cell-derived factor-1 alpha-induced lymphocyte polarization and chemotaxis. *J. Immunol.* *163*, 4001-4012.

Vicente-Manzanares,M., Rey,M., Perez-Martinez,M., Yanez-Mo,M., Sancho,D., Cabrero,J.R., Barreiro,O., de la,F.H., Itoh,K., and Sanchez-Madrid,F. (2003). The RhoA effector mDia is induced during T cell activation and regulates actin polymerization and cell migration in T lymphocytes. *J. Immunol.* *171*, 1023-1034.

Vila-Coro,A.J., Rodriguez-Frade,J.M., Martin,d.A., Moreno-Ortiz,M.C., Martinez,A., and Mellado,M. (1999). The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J.* *13*, 1699-1710.

Vlahakis,S.R., Villas-Keever,A., Gomez,T., Vanegas,M., Vlahakis,N., and Paya,C.V. (2002). G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. *J. Immunol.* *169*, 5546-5554.

Von Andrian,U.H., Hasslen,S.R., Nelson,R.D., Erlandsen,S.L., and Butcher,E.C. (1995). A central role for microvillous receptor presentation in leukocyte adhesion under flow. *Cell* *82*, 989-999.

Von Andrian,U.H. and Mackay,C.R. (2000). T-cell function and migration. Two sides of the same coin. *N. Engl. J. Med.* *343*, 1020-1034.

Vulcano,M., Albanesi,C., Stoppacciaro,A., Bagnati,R., D'Amico,G., Struyf,S., Transidico,P., Bonecchi,R., Del Prete,A., Allavena,P., Ruco,L.P., Chiabrando,C., Girolomoni,G., Mantovani,A., and Sozzani,S. (2001). Dendritic cells as a major source of macrophage-derived chemokine/CCL22 in vitro and in vivo. *Eur. J. Immunol.* *31*, 812-822.

Waas,W.F., Rainey,M.A., Szafranska,A.E., Cox,K., and Dalby,K.N. (2004). A kinetic approach towards understanding substrate interactions and the catalytic mechanism of the serine/threonine protein kinase ERK2: identifying a potential regulatory role for divalent magnesium. *Biochim. Biophys. Acta* *1697*, 81-87.

Wain,J.H., Kirby,J.A., and Ali,S. (2002). Leucocyte chemotaxis: Examination of mitogen-activated protein kinase and phosphoinositide 3-kinase activation by Monocyte Chemoattractant Proteins-1, -2, -3 and -4. *Clin. Exp. Immunol.* *127*, 436-444.

Walz,A., Peveri,P., Aschauer,H., and Baggiolini,M. (1987). Purification and amino acid sequencing of NAF, a novel neutrophil-activating factor produced by monocytes. *Biochem. Biophys. Res. Commun.* *149*, 755-761.

Wang,D., Sai,J., Carter,G., Sachpatzidis,A., Lolis,E., and Richmond,A. (2002a). PAK1 kinase is required for CXCL1-induced chemotaxis. *Biochemistry* 41, 7100-7107.

Wang,F., Herzmark,P., Weiner,O.D., Srinivasan,S., Servant,G., and Bourne,H.R. (2002b). Lipid products of PI(3)Ks maintain persistent cell polarity and directed motility in neutrophils. *Nat. Cell Biol.* 4, 513-518.

Wang,J., Arbuzova,A., Hangyas-Mihalyne,G., and McLaughlin,S. (2001). The effector domain of myristoylated alanine-rich C kinase substrate binds strongly to phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* 276, 5012-5019.

Ward,S.G. (2000). Measurement of phosphoinositide 3-kinase activity. *Methods Mol. Biol.* 138, 163-172.

Ward,S.G. and Cantrell,D.A. (2001). Phosphoinositide 3-kinases in T lymphocyte activation. *Curr. Opin. Immunol.* 13, 332-338.

Way,K.J., Chou,E., and King,G.L. (2000). Identification of PKC-isoform-specific biological actions using pharmacological approaches. *Trends Pharmacol. Sci.* 21, 181-187.

Webb,L.M., Ehrenguber,M.U., Clark-Lewis,I., Baggiolini,M., and Rot,A. (1993). Binding to heparan sulfate or heparin enhances neutrophil responses to interleukin 8. *Proc. Natl. Acad. Sci. U. S. A* 90, 7158-7162.

Weber,K.S., Weber,C., Ostermann,G., Dierks,H., Nagel,W., and Kolanus,W. (2001). Cytohesin-1 is a dynamic regulator of distinct LFA-1 functions in leukocyte arrest and transmigration triggered by chemokines. *Curr. Biol.* 11, 1969-1974.

Weiner,O.D., Neilsen,P.O., Prestwich,G.D., Kirschner,M.W., Cantley,L.C., and Bourne,H.R. (2002). A PtdInsP(3)- and Rho GTPase-mediated positive feedback loop regulates neutrophil polarity. *Nat. Cell Biol.* 4, 509-513.

Werry,T.D., Wilkinson,G.F., and Willars,G.B. (2003). Cross talk between P2Y2 nucleotide receptors and CXC chemokine receptor 2 resulting in enhanced Ca²⁺ signaling involves enhancement of phospholipase C activity and is enabled by incremental Ca²⁺ release in human embryonic kidney cells. *J. Pharmacol. Exp. Ther.* 307, 661-669.

Widmann,C., Gibson,S., Jarpe,M.B., and Johnson,G.L. (1999). Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev.* 79, 143-180.

Wilkinson,S.E., Parker,P.J., and Nixon,J.S. (1993). Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem. J.* 294 (Pt 2), 335-337.

Williams,M.R., Arthur,J.S., Balendran,A., van der,K.J., Poli,V., Cohen,P., and Alessi,D.R. (2000). The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr. Biol.* 10, 439-448.

Wing,M.R., Houston,D., Kelley,G.G., Der,C.J., Siderovski,D.P., and Harden,T.K. (2001). Activation of phospholipase C-epsilon by heterotrimeric G protein betagamma-subunits. *J. Biol. Chem.* 276, 48257-48261.

Wishart,M.J., Taylor,G.S., and Dixon,J.E. (2001). Phoxy lipids: revealing PX domains as phosphoinositide binding modules. *Cell* 105, 817-820.

Wolpe,S.D., Davatellis,G., Sherry,B., Beutler,B., Hesse,D.G., Nguyen,H.T., Moldawer,L.L., Nathan,C.F., Lowry,S.F., and Cerami,A. (1988). Macrophages secrete a novel heparin-binding protein with inflammatory and neutrophil chemokinetic properties. *J. Exp. Med.* 167, 570-581.

Wong,M., Silverman,E.D., and Fish,E.N. (1997). Evidence for RANTES, monocyte chemotactic protein-1, and macrophage inflammatory protein-1 beta expression in Kawasaki disease. *J. Rheumatol.* 24, 1179-1185.

- Wong,M., Uddin,S., Majchrzak,B., Huynh,T., Proudfoot,A.E., Plataniias,L.C., and Fish,E.N. (2001). Rantes activates Jak2 and Jak3 to regulate engagement of multiple signaling pathways in T cells. *J. Biol. Chem.* 276, 11427-11431.
- Woo,C.H., Jeong,D.T., Yoon,S.B., Kim,K.S., Chung,I.Y., Saeki,T., and Kim,J.H. (2002). Eotaxin induces migration of RBL-2H3 mast cells via a Rac-ERK-dependent pathway. *Biochem. Biophys. Res. Commun.* 298, 392-397.
- Wu,D., Huang,C.K., and Jiang,H. (2000). Roles of phospholipid signaling in chemoattractant-induced responses. *J. Cell Sci.* 113 (Pt 17), 2935-2940.
- Wu,D., LaRosa,G.J., and Simon,M.I. (1993). G protein-coupled signal transduction pathways for interleukin-8. *Science* 261, 101-103.
- Wu,D.Q., Lee,C.H., Rhee,S.G., and Simon,M.I. (1992). Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. *J. Biol. Chem.* 267, 1811-1817.
- Wurmser,A.E., Gary,J.D., and Emr,S.D. (1999). Phosphoinositide 3-kinases and their FYVE domain-containing effectors as regulators of vacuolar/lysosomal membrane trafficking pathways. *J. Biol. Chem.* 274, 9129-9132.
- Wuyts,W.A., Vanaudenaerde,B.M., Dupont,L.J., Demedts,M.G., and Verleden,G.M. (2003). Involvement of p38 MAPK, JNK, p42/p44 ERK and NF-kappaB in IL-1beta-induced chemokine release in human airway smooth muscle cells. *Respir. Med.* 97, 811-817.
- Wymann,M.P., Bjorklof,K., Calvez,R., Finan,P., Thomast,M., Trifilieff,A., Barbier,M., Altruda,F., Hirsch,E., and Laffargue,M. (2003a). Phosphoinositide 3-kinase gamma: a key modulator in inflammation and allergy. *Biochem. Soc. Trans.* 31, 275-280.
- Wymann,M.P., Zvelebil,M., and Laffargue,M. (2003b). Phosphoinositide 3-kinase signalling--which way to target? *Trends Pharmacol. Sci.* 24, 366-376.
- Xiao,T., Fujita,H., Saeki,H., Mitsui,H., Sugaya,M., Tada,Y., Kakinuma,T., Torii,H., Nakamura,K., Asahina,A., and Tamaki,K. (2003). Thymus and activation-regulated chemokine (TARC/CCL17) produced by mouse epidermal Langerhans cells is upregulated by TNF-alpha and IL-4 and downregulated by IFN-gamma. *Cytokine* 23, 126-132.
- Xu,J., Wang,F., Van Keymeulen,A., Herzmark,P., Straight,A., Kelly,K., Takuwa,Y., Sugimoto,N., Mitchison,T., and Bourne,H.R. (2003). Divergent signals and cytoskeletal assemblies regulate self-organizing polarity in neutrophils. *Cell* 114, 201-214.
- Xu,Y., Seet,L.F., Hanson,B., and Hong,W. (2001). The Phox homology (PX) domain, a new player in phosphoinositide signalling. *Biochem. J.* 360, 513-530.
- Yamasaki,M., Arai,H., Ashida,N., Ishii,K., and Kita,T. (2001). Monocyte chemoattractant protein 1 causes differential signalling mediated by proline-rich tyrosine kinase 2 in THP-1 cells. *Biochem. J.* 355, 751-756.
- Yang,C. and Kazanietz,M.G. (2003). Divergence and complexities in DAG signaling: looking beyond PKC. *Trends Pharmacol. Sci.* 24, 602-608.
- Yang,Y.M., Hatch,W.C., Liu,Z.Y., Du,B., and Groopman,J.E. (2001). beta-Chemokine induction of activation protein-1 and cyclic amp responsive element activation in human myeloid cells. *Cell Growth Differ.* 12, 211-221.
- Yoneyama,H., Harada,A., Imai,T., Baba,M., Yoshie,O., Zhang,Y., Higashi,H., Murai,M., Asakura,H., and Matsushima,K. (1998). Pivotal role of TARC, a CC chemokine, in bacteria-induced fulminant hepatic failure in mice. *J. Clin. Invest* 102, 1933-1941.

Yoshida,T., Izawa,D., Nakayama,T., Nakahara,K., Kakizaki,M., Imai,T., Suzuki,R., Miyasaka,M., and Yoshie,O. (1999). Molecular cloning of mXCR1, the murine SCM-1/lymphotactin receptor. *FEBS Lett.* **458**, 37-40.

Yoshie,O., Fujisawa,R., Nakayama,T., Harasawa,H., Tago,H., Izawa,D., Hieshima,K., Tatsumi,Y., Matsushima,K., Hasegawa,H., Kanamaru,A., Kamihira,S., and Yamada,Y. (2002). Frequent expression of CCR4 in adult T-cell leukemia and human T-cell leukemia virus type 1-transformed T cells. *Blood* **99**, 1505-1511.

Yoshimura,T., Matsushima,K., Oppenheim,J.J., and Leonard,E.J. (1987a). Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *J. Immunol.* **139**, 788-793.

Yoshimura,T., Matsushima,K., Tanaka,S., Robinson,E.A., Appella,E., Oppenheim,J.J., and Leonard,E.J. (1987b). Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc. Natl. Acad. Sci. U. S. A* **84**, 9233-9237.

Youn,B.S., Mantel,C., and Broxmeyer,H.E. (2000). Chemokines, chemokine receptors and hematopoiesis. *Immunol. Rev.* **177**, 150-174.

Youn,B.S., Yu,K.Y., Oh,J., Lee,J., Lee,T.H., and Broxmeyer,H.E. (2002). Role of the CC chemokine receptor 9/TECK interaction in apoptosis. *Apoptosis.* **7**, 271-276.

Yu,D.H., Qu,C.K., Henegariu,O., Lu,X., and Feng,G.S. (1998a). Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. *J. Biol. Chem.* **273**, 21125-21131.

Yu,J., Zhang,Y., McIlroy,J., Rordorf-Nikolic,T., Orr,G.A., and Backer,J.M. (1998b). Regulation of the p85/p110 phosphatidylinositol 3'-kinase: stabilization and inhibition of the p110alpha catalytic subunit by the p85 regulatory subunit. *Mol. Cell Biol.* **18**, 1379-1387.

Yule,D.I. and Williams,J.A. (1992). U73122 inhibits Ca²⁺ oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. *J. Biol. Chem.* **267**, 13830-13835.

Yumura,S., Furuya,K., and Takeuchi,I. (1996). Intracellular free calcium responses during chemotaxis of Dictyostelium cells. *J. Cell Sci.* **109** (Pt 11), 2673-2678.

Yusufi,A.N., Cheng,J., Thompson,M.A., Burnett,J.C., and Grande,J.P. (2002). Differential mechanisms of Ca(2+) release from vascular smooth muscle cell microsomes. *Exp. Biol. Med.* (Maywood.) **227**, 36-44.

Zabel,B.A., Agace,W.W., Campbell,J.J., Heath,H.M., Parent,D., Roberts,A.I., Ebert,E.C., Kassam,N., Qin,S., Zovko,M., LaRosa,G.J., Yang,L.L., Soler,D., Butcher,E.C., Ponath,P.D., Parker,C.M., and Andrew,D.P. (1999). Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. *J. Exp. Med.* **190**, 1241-1256.

Zebda,N., Bernard,O., Bailly,M., Welti,S., Lawrence,D.S., and Condeelis,J.S. (2000). Phosphorylation of ADF/cofilin abolishes EGF-induced actin nucleation at the leading edge and subsequent lamellipod extension. *J. Cell Biol.* **151**, 1119-1128.

Zemlickova,E., Johannes,F.J., Aitken,A., and Dubois,T. (2004). Association of CPI-17 with protein kinase C and casein kinase I. *Biochem. Biophys. Res. Commun.* **316**, 39-47.

Zernecke,A., Weber,K.S., and Weber,C. (2001). Combined modulation of the mesangial machinery for monocyte recruitment by inhibition of NF-kappaB. *Am. J. Physiol Cell Physiol* **281**, C1881-C1888.

Zhan,Q., Bamberg,J.R., and Badwey,J.A. (2003). Products of phosphoinositide specific phospholipase C can trigger dephosphorylation of cofilin in chemoattractant stimulated neutrophils. *Cell Motil. Cytoskeleton* **54**, 1-15.

Zhang,S., Lukacs,N.W., Lawless,V.A., Kunkel,S.L., and Kaplan,M.H. (2000). Cutting edge: differential expression of chemokines in Th1 and Th2 cells is dependent on Stat6 but not Stat4. *J. Immunol.* 165, 10-14.

Zhang,X.F., Wang,J.F., Matczak,E., Proper,J.A., and Groopman,J.E. (2001). Janus kinase 2 is involved in stromal cell-derived factor-1alpha- induced tyrosine phosphorylation of focal adhesion proteins and migration of hematopoietic progenitor cells. *Blood* 97, 3342-3348.

Zhang,Y., Zhai,Q., Luo,Y., and Dorf,M.E. (2002). RANTES-mediated chemokine transcription in astrocytes involves activation and translocation of p90 ribosomal S6 protein kinase (RSK). *J. Biol. Chem.* 277, 19042-19048.

Zheng,X., Nakamura,K., Furukawa,H., Nishibu,A., Takahashi,M., Tojo,M., Kaneko,F., Kakinuma,T., and Tamaki,K. (2003). Demonstration of TARC and CCR4 mRNA expression and distribution using in situ RT-PCR in the lesional skin of atopic dermatitis. *J. Dermatol.* 30, 26-32.

Zou,Y.R., Kottmann,A.H., Kuroda,M., Taniuchi,I., and Littman,D.R. (1998). Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 393, 595-599.

8

GLOSSARY

8. GLOSSARY

Accessory cell	Cell required for, but not actually mediating, a specific immune response. Often used to describe antigen-presenting cells (APC; see below).
Acid	A molecule that releases H ⁺ ion (proton).
Acquired immune response	Immunity mediated by lymphocytes and characterized by antigen-specificity and memory.
Active transport	Movement of a molecule across a membrane or other barrier driven by energy other than that stored in the concentration gradient or electrochemical gradient of the transported molecule.
Adjuvant	Any substance that nonspecifically enhances the immune response to antigen.
Affinity (intrinsic affinity)	The strength of binding (affinity constant) between a receptor (e.g. one antigen-binding site on an antibody) and a ligand (e.g. epitope on an antigen).
Agglutination	The aggregation of particulate antigen by antibodies. Agglutination applies to red blood cells as well as to bacteria and inert particles covered with antigen.
Akt/PKB	65 kDa serine/threonine protein kinase, also known as protein kinase B (PKB). Critical mediator of cell survival and major effector of PI3K activity.
Allele	Variants of a polymorphic gene at a given genetic locus; alternative forms of a genetic locus; a single allele for each locus is inherited separately from each parent (e.g. at a locus for eye color the allele might result in blue or brown eyes).
Allelic	Relating to one of a series of two or more alternate forms of a gene that occupy the same position or locus on a specific chromosome.
Allelic exclusion	The phenomenon whereby, following successful rearrangement of one allele of an antigen receptor gene, rearrangement of the other parental allele is suppressed, thereby ensuring each lymphocyte expresses only a single specificity of antigen receptor.
Allergen	An antigen responsible for producing allergic reactions by inducing IgE formation.
Allergy	'Changed reactivity' of the host when meeting an 'agent' on a second or subsequent occasion mediated by IgE - IgE-mediated hypersensitivity, e.g. asthma, eczema, hay fever and food allergy.
Allogeneic	The allelic variation (genetic differences) observed among members of the same species.
Allograft	A tissue transplant (graft) between two genetically non-identical (allogenic) members of a species.
Alloreactive	Reactive with foreign tissue type.
Allotypes:	Antigenic determinants that are present in allelic (alternate) forms. When used in association with immunoglobulin, allotypes describe allelic variants of immunoglobulins detected by antibodies raised between members of the same species (i.e. An allelic variant of an antigen, which, because it is not present in all individuals, may be immunogenic in members of the same species which have a different version of the allele).
Amino acid	Any of a class of 20 molecules that are combined to form proteins in living things. The sequence of amino acids in a protein and hence protein function are determined by the genetic code. Consist of both a carboxylic acid group as well as an amino acid group.
Anaphylatoxin	A substance (e.g. C3a, C4a or C5a) capable of directly triggering mast cell degranulation (histamine release).
Anaphylaxis:	An often fatal immediate hypersensitivity reaction to antigenic challenge, triggered by IgE or anaphylatoxin-mediated mast cell degranulation, leading to anaphylactic shock due to vasodilatation and smooth muscle contraction.
Anergy	Potentially reversible specific immunological tolerance in which the lymphocyte becomes functionally nonresponsive.
Antibody	Serum protein (immunoglobulin) formed in response to immunization, composed of constant and variable regions. The variable region has a special configuration that matches that of the antigen and binds to it, the constant region helps to eliminate it. Antibodies are generally defined in terms of their specific binding to the immunizing antigen.
Antibody-dependent, cell-mediated cytotoxicity (ADCC)	A phenomenon in which target cells, coated with antibody, are destroyed by specialized killer cells (NK cells, neutrophils and macrophages), which bear receptors for the Fc portion of the coating antibody (Fc receptors). These receptors allow the killer cells to bind to the anti-body-coated target.
Antigen	Any molecule capable of being recognized by an antibody or T-cell receptor. An antigen is a substance that can trigger an immune response. Many antigens are foreign proteins. An allergen is a special type of antigen that causes an IgE antibody response. Antigens may also be immunogens if they are able to trigger an immune response (resulting in production of an antibody as part of the body's defence against infection and disease), or haptens if not.
Antigen presenting cell (APC)	A specialized type of cell, bearing cell surface class II MHC (major histocompatibility complex) molecules, involved in processing and presentation of processed antigenic peptide to the T cell receptor on CD4 T cells, e.g. macrophages, dendritic cells, B cells. Note, however, that most types of cell are able to present antigenic peptides with MHC class I to CD8 T-cells, e.g. as occurs with virally infected cells.

Antigen processing	Large molecules are broken down (processed) within macrophages into peptides and presented within the groove of MHC molecules.
Antigen receptor	The specific antigen-binding receptor on T or B lymphocytes; these receptors are transcribed and translated from rearrangements of V genes.
Antigen-binding site	The part of an immunoglobulin molecule that binds antigen specifically.
Antigenic determinant	A single antigenic site or epitope on a complex antigenic molecule or particle; a cluster of epitopes (see 'epitope').
Antisense	The non-coding strand in double-stranded DNA. The antisense strand serves as the template for mRNA synthesis.
Apoptosis	Programmed cell death ("suicide") as signaled by the nuclei (and characterized by endonuclease digestion of DNA) in normally functioning human and animal cells when age or state of cell health and condition dictates.
A-Raf	68 kDa cytoplasmic serine/threonine protein kinase that is activated by Ras-GTP and phosphorylation.
Asthma	Asthma is a chronic, inflammatory lung disease characterized by recurrent breathing problems. People with asthma have acute episodes (when the air passages in their lungs get narrower, and breathing becomes more difficult). Sometimes episodes of asthma are triggered by allergens, although infection, exercise, cold air and other factors are also important triggers.
atopic allergy	IgE-mediated hypersensitivity, i.e. asthma, eczema, hay fever and food allergy.
Atopy	A term used to describe IgE-mediated anaphylactic responses in humans, usually genetically determined.
ATP (adenosine triphosphate)	A triphosphate ester of adenine that participates in the transfer of energy in hundreds of individual cellular reactions.
Autograft	A tissue transplant from one area to another on a single individual.
Autoimmune disease	A disease caused by the immune system "rejecting" self tissues.
Autoimmunity (autoallergy)	An immune response to "self" tissues or components. Such an immune response may have pathological consequences leading to autoimmune diseases.
Autologous	From the same individual.
Autosomal dominant	A pattern of Mendelian inheritance whereby an affected individual possesses one copy of a mutant allele and one normal allele. In contrast, recessive diseases require that the individual have two copies of the mutant allele. Individuals with autosomal dominant diseases have a 50-50 chance of passing the mutant allele and hence the disorder onto their children.
Autosome	Any chromosome other than a sex chromosome.
Avidity (functional affinity)	The binding strength between two molecules (e.g. antibody and antigen) taking into account the valency of the interaction (i.e. the summation of multiple affinities – e.g. when a polyvalent antibody binds to a polyvalent antigen). Thus the avidity will always be equal to or greater than the intrinsic affinity (<i>see</i> affinity).
B cell antigen receptor	Membrane-bound immunoglobulin on the surface of B cells that specifically recognizes intact antigen and triggers a B cell immune response.
B cells	Lymphocytes that are produced in the bone marrow. When B cells are stimulated by antigens, they are converted into antibody-forming plasma cells; these cells carry immunoglobulin and class II MHC (major histocompatibility complex) antigens on their surfaces.
Bad	A 23 kDa member of the Bcl-2 family that is pro-apoptotic.
Base	A molecule that accepts H ⁺ ion (proton).
Base pair	Two nitrogenous bases (adenine and thymine or guanine and cytosine) held together by weak bonds. Two strands of DNA are held together in the shape of a double helix by the bonds between base pairs.
Basophil	A polymorphonuclear leukocyte (and a type of granulocyte), whose granules contain heparin, histamine and other vasoactive amines. Within tissues, these cells are known as mast cells.
BCR/ABL	210/190 kDa A hybrid tyrosine kinase that is a product of the fusion between c-ABL and the BCR gene.
β-arrestin	A member of the arrestin family, which has been implicated in mediating the desensitization and internalization of GPCRs. Constitutively phosphorylated by ERK1/2 and localized in the cytosol. When recruited to the plasma membrane after agonist stimulation, β-arrestin is rapidly dephosphorylated – essential for receptor internalization but not desensitization.
Beta 2-microglobulin	A 12kDa protein, not itself encoded within the MHC, but forming part of the structure of MHC class I-encoded molecules.
Beta-pleated sheet	A planar secondary structure element of proteins that is created by hydrogen bonding between the backbone atoms in two different polypeptide chains or segment of a single folded chain.
Bispecific antibody	An artificially produced hybrid antibody in which each of the two antigen-binding arms is specific for a different antigenic epitope. Such antibodies, which can be produced either by chemical cross-linkage or by recombinant DNA techniques, can be used to link together two different antigens or cells, e.g. a cytotoxic T-cell and a tumor cell.
Blocking antibody	A functional term for an antibody molecule capable of blocking the interaction of antigen/stimulant with other antibodies or with cells.
Bone marrow	Blood-cell producing tissue inside the bones that makes the red (erythrocytes) and white (leukocytes) blood cells.

B-raf	68-95 kDa member of the Raf family of serine/threonine kinases expressed primarily in the brain and in the nervous system. A MAPK kinase kinase (MAPKKK).
Btk	77 kDa Bruton's Tyrosine Kinase (Btk); a member of the Tec family, is a cytoplasmic protein tyrosine kinase that is phosphorylated and activated after antigen stimulation of the BCR.
Cadherin	120 kDa transmembrane protein involved in cellular adhesions.
Cak-beta (RAFTK/PYK2)	99-125 kDa cell adhesion kinase beta (also known as RAFTK, FAK2 or Pyk2); a protein tyrosine kinase that is part of the Focal Adhesion Kinase (FAK) family. It is involved in many signaling cascades linking cell surface events to the cytoskeleton.
Calmodulin	~22 kDa intracellular calcium-binding protein consisting of at least two different peptide chains.
Calpain	A member of the family of Ca(2+)-dependent intracellular 28 kDa cysteine proteases.
CaM kinase II	50-60 kDa calmodulin-dependent protein kinase II; a serine/threonine kinase that is activated in the presence of increased Ca(2+).
cAMP	Adenosine 3'5' cyclic monophosphate; a second messenger with a role in gene transcription, neuronal functions, and cell proliferation. Also regulates GSK-3 and transcription factors such as STAT.
Capping	An active process whereby cross-linking of cell surface molecules (e.g. by antibody) leads to aggregation and subsequent migration of the molecules to one pole of the cell.
Carrier	Any molecule which when conjugated to a non-immunogenic molecule (e.g. a hapten) makes the latter immunogenic by providing epitopes for helper T-cells which the hapten lacks.
Carrier proteins	Membrane transport protein that binds to a solute and transports it across the membrane by undergoing a series of conformational changes.
Caspase	<i>CYSTEINE PROTEASES WITH ASPARTATE SPECIFICITY.</i>
Caspase-2	49 kDa cysteine aspartyl protease-2; an initiator caspase for apoptosis. Also known as ICH-1 and NEDD-2
Caspase-3	33 kDa cysteine aspartyl protease-3; an effector caspase for apoptosis. Also known as LICE, CPP32, YAMA, apopain and SCA-1.
Caspase-8	55 kDa cysteine aspartyl protease-8; an initiator caspase for apoptosis. Also known as MACH, FLICE, and Mch5.
Caspase-9	59 kDa cysteine aspartyl protease-9; an initiator caspase for apoptosis. Also known as ICE-LAP6, Mch6, and APAF-3.
CD	"Cluster Designation/Differentiation": international nomenclature for leukocyte cell surface molecules which are identified by a given group of monoclonal antibodies (CD number).
CD25	The alpha chain of the IL-2 receptor expressed on activated T cells.
CD28	A co-stimulatory molecule on the surface of T lymphocytes that signals activation by binding to B7 molecules on antigen presenting cells.
CD3	A trimeric complex of γ , δ and ϵ chains which together with a $\zeta\zeta$ or $\eta\eta$ homodimer, or a $\zeta\eta$ heterodimer acts as a signal transducing unit for the T-cell receptor.
CD4	Cell surface glycoprotein, usually on helper T-cells that recognizes MHC class II molecules on antigen-presenting cells (acts as a T cell signalling/co-receptor molecule).
CD40	A cell surface molecule on B cells and APCs that receives a signal for T cell help (via CD40Ligand).
CD45	A transmembrane tyrosine phosphatase that is constitutively bound to CD45AP (CD45-associated protein). CD45 controls TCR signaling, regulates tyrosine phosphorylation of CD22 and mediates activation of SHP-1; Leukocyte common antigen
CD8	Cell surface glycoprotein, usually on cytotoxic T-cells, that recognizes MHC class I molecules on target cells (acts as a T cell signalling/co-receptor molecule).
CDK1 (cdc2)	34 kDa cyclin-dependent kinase-1; a serine/threonine protein kinase that is the catalytic sub-unit of cyclin B, controlling the onset of mitosis.
CDK2	33 kDa cyclin dependent kinase-2; associates primarily with Cyclin A during G1-S transition.
CDK4	36 kDa cyclin dependent kinase-4; associates with Cyclin D1, D2 and D3 and phosphorylates the Rb protein
CDK5	31 kDa Cyclin dependent kinase-5; found in active form only in neuronal cells. Activated by association with cyclin-like neuronal proteins.
cDNA (complementary DNA)	DNA molecule made as a copy of mRNA and therefore lacking the introns that are present in genomic DNA. cDNA is synthesized from an RNA template using reverse transcriptase.
Cell line	A culture of a particular type of cell that can be reproduced indefinitely, thus making the cell line "immortal".
Cell-mediated cytotoxicity (CMC)	Killing (lysis) of a target cell by an effector lymphocyte.
Cell-mediated immunity (CMI)	Immune reaction mediated by T cells; in contrast to humoral immunity, which is antibody mediated. Also referred to as delayed-type hypersensitivity.
Cell-mediated response	The activation of T-cells, B-cells and other cells involved in the immune response.
c-fos	A short-lived transcription factor that is a member of the activator protein-1 (AP-1) family. It is activated by beta-catenin, intracellular signaling cascades, actin disassembly, RSK, and MAPK.

Channel proteins	Form hydrophilic pores that extend across the lipid bilayer; when these pores open, they allow specific molecules to pass through them
Chemokines	A family of structurally-related chemotactic cytokines which selectively induce chemotaxis and activation of leukocytes (and other cells). They also play important roles in lymphoid organ development, cell compartmentalization within lymphoid tissues, Th1/Th2 development, angiogenesis and wound healing.
Chemotaxis	Migration of cells along (up) a concentration gradient of an attractant (chemotactic factors).
Chimera	An organism or recombinant DNA molecules created by joining DNA fragments from two or more different organisms.
Chimeric	Composite of genetically distinct individuals, e.g. following an allogeneic bone marrow graft.
Chromosomes	The self-replicating genetic structures of cells containing the cellular DNA that bears in it nucleotide sequence (the linear array of genes), and thus carries part (or all) of that hereditary information of an organism.
c-Jun	36 kDa short-lived transcription factor that is a member of the activator protein-1 (AP-1) family. It has been found to both induce and inhibit apoptosis and promote trophic changes in peripheral organs.
Class I, II and III MHC molecules	Proteins encoded by genes in the major histocompatibility complex (q.v.). Class I molecules are designated HLA-A, B, or C. Class II molecules are designated DP, DQ or DR: Class I MHC - found on all nucleated cells, "self molecules"; Class II MHC - found on macrophages, B and T cells.
Class switching	The process by which a B-cell changes the class but not specificity of a given antibody it produces, e.g. switching from an IgM to an IgG antibody.
Clonal deletion	A process by which contact with antigen (i.e. self antigen or artificially introduced antigen) at an early stage of lymphocyte differentiation leads to the cell death by apoptosis of lymphocytes of a particular specificity.
Clonal selection theory	The prevalent concept that specificity and diversity of an immune response are the result of selection by antigen of specifically reactive clones from a large repertoire of preformed lymphocytes, each with individual specificities.
Clone	A genetically homogeneous population derived from a single cell (progenitor).
Cloning	The process of asexually producing a group of cells (clones), all genetically identical, from a single ancestor. In recombinant DNA technology, the use of DNA manipulation procedures to produce multiple copies of a single gene or segment of DNA is referred to as cloning DNA.
Cloning vector	DNA molecule originating from a virus, a plasmid, or the cell of a higher organism into which another DNA fragment of appropriate size can be integrated without loss of the vectors capacity for self-replication; vectors introduce foreign DNA into host cells, where it can be reproduced in large quantities. Examples are plasmids, cosmids, and yeast artificial chromosomes; vectors are often recombinant molecules containing DNA sequences from several sources.
c-myc	A transcription factor that is involved in the mitogenic signaling pathway, apoptosis, and is believed to regulate telomerase activity; a cellular homologue of avian myelocytomatosis virus oncogene.
Codon	Sequence of three nucleotides in a DNA or messenger RNA sequence that represents the instruction for incorporation of a specific amino acid into a growing polypeptide chain.
Cofilin	Cofilin and ADF (actin depolymerisation factor) are members of a family of actin-binding proteins that play pivotal roles in cytokinesis and possibly chemotaxis. Cofilin promotes the regeneration of actin filaments by severing pre-existing filaments, and nucleating new actin polymers. Activity of cofilin is inhibited by LIMK.
Cognate	Related in nature.
Combinatorial joining	The joining of segments of DNA to generate essentially new genetic information, as occurs with Ig genes during the development of B cells. Combinatorial joining allows multiple opportunities for 2 sets of genes to combine in different ways.
Complementarity determining regions (CDR)	The hypervariable amino acid sequences within antibody and T-cell receptor variable regions which interact with complementary amino acids on the antigen or peptide-MHC complex.
Complementary base pairs	Base-pairing between a larger purine base (adenine or guanine) and a smaller pyrimidine base (cytosine or thymine) while DNA is in its double-helix. (A/T, G/C)
ConA (concanavalin A)	A T-cell mitogen.
Conjugate	Covalently-linked complex of two or more molecules (e.g. fluorescein conjugated to antibody).
Connective tissues	Any supporting tissue that lies between other tissues and consists of cells embedded in a relatively large amount of extra-cellular matrix. Functions include support, storage, and protection.
Constant region (C region)	The invariant carboxyl-terminal portion of an antibody molecule, as distinct from the variable region which is at the amino-terminal of the chain.
Contact dermatitis	Contact dermatitis is an inflammation of the skin or a rash caused by contact with various substances of a chemical, animal or vegetable nature. The reaction may be an immunologic response or a direct toxic effect of the substance. Among the more common causes of a contact dermatitis reaction are detergents left on washed clothes, nickel (in watch straps, bracelets and necklaces, and the fastenings on underclothes), chemicals in rubber gloves and condoms, certain cosmetics, plants such as poison ivy, and topical medications.
Copy number	The number of copies of a transgene that is integrated into the host genome.
Co-receptor	Additional receptor molecules that contribute to the T or B cell receptor signalling complex.
Co-stimulation	Signals in addition to those through the antigen receptor that are required for full T lymphocyte activation.

COX-2	70 kDa cyclooxygenase-2 (also known as prostaglandin-endoperoxide synthase-2); an "emergency enzyme" that functions to repair and defend cells.
c-Raf	See Raf.
CREB	45 kDa cAMP response element-binding transcription factor.
Crk	40 kDa SH2 and SH3 domain-containing adaptor protein.
Cross-reactivity	The ability of an antibody, specific for one antigen, to react with a second antigen; a measure of relatedness between two different antigenic substances.
CSK	60 kDa Carboxyl-terminal Src Kinase (Csk); a cytosolic protein tyrosine kinase that functions to phosphorylate and inactivate Src family kinases.
CTLA-4	A molecule that competes with CD28 reducing costimulation (also known as CD152).
Cyclic AMP (cAMP)	Nucleotide that is generated from ATP in response to hormonal stimulation of cell-surface receptors; serves as a universal signaling molecule; a secondary messenger in signaling pathways.
Cytokines	Low molecular weight secreted and soluble proteins that stimulate or inhibit the differentiation, proliferation or function of immune cells.
Cytophilic	Binds to cells.
Cytoplasm	Contents of a cell that are contained within its plasma membrane but, in the case of eucaryotic cells, outside the nucleus.
Cytoskeleton	System of protein filaments in the cytoplasm of a eucaryotic cell that gives the cell shape and the capacity for directed movement.
Cytosol	Contents of the main compartment of the cytoplasm, excluding membrane-bounded organelles such as endoplasmic reticulum and mitochondria.
Cytotoxic	Kills cells.
Cytotoxic T lymphocyte (Tc)	T-cells (usually CD8) which kill target cells following recognition of foreign peptide (antigen) within the groove of an MHC class I molecule on the target cell membrane.
D gene	A small segment of immunoglobulin heavy-chain and T-cell receptor DNA, coding for the third hypervariable region of most receptors.
DAG	1,2-diacyl-n-glycerol (DAG); an intracellular second messenger, is found in the phospholipid bilayer of the cell membrane and controls lipid structure and PKC and phospholipase activation.
Delayed type hypersensitivity (DTH)	A T cell-mediated reaction to antigen, which takes 24-48 hours to develop fully, and which involves release of cytokines from T cells and recruitment of monocytes and macrophages. Also called cell-mediated immunity.
Dendritic cell	A cell with finger-like processes specialised for antigen presentation.
Determinant	Part of the antigen molecule that binds to an antibody-combining site or to a receptor on T cells (see hapten and epitope).
Differentiation antigen	A cell surface antigenic determinant found only on cells of a certain lineage and at a particular developmental stage; used as an immunologic marker.
Diploid	The number of chromosomes in most cells except the gametes. In humans, the diploid number is 46.
Disulfide bond	Covalent intrachain bonds found in protein molecules; covalent linking of two -SH groups of neighboring cysteine residues in a folded polypeptide chain. These bonds are rarely, if ever found in the cytosol.
Diversity (D) gene segments	Found in the immunoglobulin heavy chain gene and T-cell receptor α and β gene loci between the V and J gene segments. Encode part of the third hypervariable region in these antigen receptor chains.
DNA (deoxyribonucleic acid)	The molecule that encodes genetic information. DNA is a double-stranded molecule held together by weak bonds between base pairs of nucleotides. The four nucleotides in DNA contain the bases: (A), guanine (G), cytosine (C), and thymine (T). In nature, base pairs form only between A and T and between G and C; thus the base sequence of each single strand can be deduced from that of its partner.
DNA construct	DNA sequence that has been altered by design for the addition of a transgene, targeted mutation, replacement, or any one of a number of methods that yield a recombinant DNA sequence.
DNA helicase	An enzyme that participates in DNA replication by unwinding the double helix near the replication fork.
DNA ligase	Fills in nicks and gaps made in polynucleotide strands.
DNA polymerase	Catalyzes the addition, of a deoxyribonucleotide to the 3' end of a DNA chain.
DNA renaturation (hybridization)	Process whereby two complementary nucleic acid strands form a double helix during an annealing period; a powerful technique for detecting specific nucleotide sequences.
DNA topoisomerases	Enzymes that make reversible cuts in a double helical DNA molecule for the purpose of removing knots or unwinding excessive twists.
DNA transcription	Process in which molecules of RNA are synthesized in which a strand of DNA acts as a template.
p62Dok	A major tyrosine-phosphorylated, GAP-associated protein present in cells transformed by different tyrosine kinases. Overexpression has been shown to inhibit Ras activity.
Domain	Certain combinations of helices and sheets which pack together to form a compactly folded globular unit.
4E-BP1	Normally binds eIF4E, inhibiting cap-dependent translation. Regulated by the PI3K-PKB and mTOR pathway(s).
Effector cell	A cell that has developed full immune functions, such as cytokine release and cytotoxicity (eg cytotoxic/killer cell)

Electrophoresis	Separation technique in which an electric field is applied to a solution containing a protein molecule; the protein will migrate at a rate depending on its net charge and on its size and shape.
Electroporation	The exposure of cells to rapid pulses of high-voltage current which renders the plasma membrane of the cells permeable and thus allowing transfection.
ELK-1	A transcription factor and component of the ternary complex that binds SRE and mediates gene regulation in response to serum and growth factors. Activated by ERK.
Embryonic stem cells (ES cells)	A cell of the early embryo that can replicate indefinitely and which can differentiate into other cells; stem cells serve as a continuous source of new cells.
Endocrine cell	Specialized animal cell that secretes a hormone into the blood; usually part of a gland, such as the thyroid or pituitary gland.
Endocytosis	Cellular ingestion of macromolecules by invagination of plasma membrane to produce an intracellular vesicle which encloses the ingested material.
Endogenous	From within.
Endonucleases	Enzymes that hydrolyze internal phosphodiester bonds in a polynucleotide chain or nucleic acid molecule.
Endoplasmic reticulum	Labyrinthine, membrane-bounded compartment in the cytoplasm of eucaryotic cells, where lipids are synthesized and membrane-bound proteins are made.
Endosomes	Intracellular smooth surfaced vesicles in which endocytosed material passes on its way to the lysosomes.
Endotoxin	Pathogenic cell wall-associated lipopolysaccharides of Gram-negative bacteria.
Enzymes	Highly specific protein catalysts, they speed up the reactions by reducing the activation energy for a particular chemical change.
Eosinophil	A class of granulocyte (and polymorphonuclear leukocyte), the granules of which contain toxic cationic proteins.
Epithelial tissues (epithelium)	Cells tightly bound together into sheets.
Epitope	An alternative term for antigenic determinant.
ERK1/2 (MAPK)	44/42 kDa Extracellular signal-Regulated protein Kinase. ERK1 (p44mapk) and ERK2 (p42mapk) are proline-directed protein kinases that are activated by dual phosphorylation (pT x pY motif) and catalyzed by MEKs. They regulate transcription, cell cycle, differentiation, and learning and memory. ERK3 (p62mapk) is believed to be a unique ERK with distinct functional roles. ERK4 plays a role in intracellular signaling via phosphorylation by Ras. ERK5 is stimulated by oxidative stress and helps control c-jun expression.
Erythropoiesis	Erythrocyte production.
Exon	The region of a gene that contains the code for producing the gene's protein. Each exon codes for a specific portion of the complete protein.
Exotoxin	Pathogenic protein secreted by bacteria.
Extrinsic asthma	Extrinsic asthma is asthma that is triggered by an allergic reaction, usually something that is inhaled.
Exudate	The extravascular fluid (containing proteins and cellular debris) which accumulates during inflammation.
Ezrin	Ezrin, radixin and moesin (ERM) proteins function as linkers between the plasma membrane and the actin cytoskeleton, and are involved in cell adhesion, membrane ruffling and microvilli formation.
F(ab')₂	A Bivalent antigen-binding fragment of an antibody containing two antigen-binding sites generated by cleavage of the antibody molecule with the enzyme pepsin which cuts at the hinge region C-terminally to the inter-H-chain disulphide bond. Thus, consists of both light chains and the N-terminal part of both heavy chains linked by disulfide bonds.
F1 hybrids	The first generation of mice generated from 2 different inbred strains. These mice are genetically identical to one another but different from either inbred parent.
F2 hybrids	The progeny produced from matings between F1 mice. These mice are different from one another and will contain different mixtures of the genetic variations that were present in the original inbred progenitors.
Fab	A monovalent antigen-binding fragment of antibody containing the antigen-binding site, generated by cleavage of the antibody with the enzyme papain, which cuts at the hinge region N-terminally to the inter-H-chain disulphide bond and generates two Fab fragments from one antibody molecule. Consists of an intact light chain and the N-terminal V _H and C _H 1 domains of the heavy chain.
F-actin	42 kDa focal adhesion protein that links to integrins.
FADD	23 kDa Fas-associated Death Domain; an adaptor protein that mediates apoptosis from receptor to protease.
FAK	125 kDa Focal Adhesion Kinase (pp125FAK) is a non-receptor tyrosine kinase that functions in integrin- and growth factor-mediated signaling and is involved in regulating cell adhesion, migration, and cell cycle events.
Fas	A member of the TNF receptor gene family. Engagement of Fas (CD95) on the surface of the cell by the Fas ligand (CD178) present on cytotoxic cells, can trigger apoptosis in the Fas-bearing target cell.
Fc	Crystallizable, non-antigen binding fragment of an immunoglobulin molecule obtained following papain digestion. Consists of the C-terminal portion of both heavy immunoglobulin chains, which is responsible for binding to Fc receptors and C1q.
Fc receptor (FcR)	A receptor on a cell surface with specific binding affinity for the Fc portion of particular immunoglobulin classes. Fc receptors are found on many types of cells.
Fibroblast	Connective tissue cell, which produces collagen and plays an important part in wound healing.

FKHR	A member of the Forkhead family of transcription factors involved in cell survival and cell cycle regulation.
Flanking region	The DNA sequences extending on either side of a specific locus or gene.
Fluorescein isothiocyanate (FITC)	Green flu-orescent dye used to 'tag' antibodies for use in immunofluorescence.
Fluorescent antibody	An antibody coupled with a fluorescent dye (e.g. FITC), used with a fluorescence microscope to detect antigen on cells, tissues, or microorganisms.
Follicular dendritic cell	MHC class II-negative Fc receptor-positive dendritic cells which bear immune complexes on their surface and are probably involved in the generation of antibody-secreting cells and maintenance of B-cell memory in germinal centres. (N.B. a different cell type to interdigitating dendritic cells).
Fos B	46 kDa FBI osteosarcoma virus, an AP-1 family member, associated with tumors expressing Rb.
Gαi	Family of G protein subunits that can inhibit adenylyl cyclase.
Gαq	Family of G protein subunits that controls the activity of PI-specific phospholipases.
Gαs	Family of G protein subunits that can stimulate adenylyl cyclases.
Gab 1	110 kDa Grb2-associated binder 1; an IRS-related adaptor protein that forms a complex with SHP2 and PI3kinase. Promotes both ERK and Elk-1 activation.
Gab 2	100 kDa Grb2-associated binder 2; an IRS-related adaptor protein that associates with SHP2 and Grb2. It can activate ERK but negatively regulates Elk-1.
Gamma-globulin	The serum proteins, mostly immunoglobulins, which have the greatest mobility towards the cathode during electrophoresis.
GEF	Guanine nucleotide Exchange Factor.
Gene	The fundamental physical and functional unit of heredity. A gene is an ordered sequence of nucleotides located in a particular position on a particular chromosome that encodes a specific functional product (i.e., a protein or RNA molecule). See gene expression.
Gene expression	The process by which a gene's coded information is converted into the structures present and operating in the cell. Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated into protein (e.g., transfer and ribosomal RNAs).
Gene mapping	Determination of the relative positions of genes on a DNA molecule (chromosome or plasmid) and of the distance, in linkage units or physical units, between them.
Gene targeting	The creation of a null or mutant allele by homologous recombination or gene replacement.
Gene therapy	The process of introducing genes into a person's cells to correct a disease or genetic flaw. This technique has been useful in developing animal models for a variety of genetic disorders (i.e. growth, immunological, neurological, reproductive, and hematological disorders).
Gene trap	Vectors that carry the reporter gene (with or without the start codon ATG) 3' to a splice acceptor site (a splice acceptor site is used instead of a promoter). Used primarily in ES cells to screen for genes expressed during mouse embryogenesis.
Genome	All the genetic material in the chromosomes of a particular organism; its size is generally given as its total number of base pairs.
Genotype	All of the genes possessed by an individual; in practice it refers to the particular alleles present at the loci in question.
Germinal center	Discrete areas within lymph node and spleen where B-cell maturation and memory development occur.
Giant cell	Large multinucleate cell derived from fused macrophages and often present in granulomas.
Glial cells	Supporting cells of the nervous system, including oligodendrocytes and astrocytes in the vertebrate central nervous system and Schwann cells in the peripheral nervous system.
Glomerulonephritis	Inflammation of renal glomerular capillary loops, often resulting from immune complex deposition.
Glycogen	Polysaccharide composed exclusively of glucose units used to store energy in animal cells. Large granules of glycogen are especially abundant in the liver and muscle cells.
Glycolipid	Membrane lipid molecule with a short carbohydrate chain attached to a hydrophobic tail.
Glycolysis	The degradation of carbohydrates in a sequence of enzymatically catalyzed steps.
Glycoprotein	Any protein with one or more covalently linked oligosaccharide chains. Included most secreted proteins and most proteins exposed on the outer surface of the plasma membrane.
Glycosidic bond	The carbon that carries the aldehyde or the ketone can react with any hydroxyl group on a second sugar molecule to form a glycosidic bond.
Golgi apparatus	Membrane-bounded organelle in eucaryotic cells where the proteins and lipids made in the endoplasmic reticulum are modified and stored.
Graft rejection (GVHD)	When immunologically competent graft is transported into an immunologically compromised host, the graft tissue (containing T cells) can mount an immunologic attack on the recipient.
Granulocyte	Myeloid cells containing cytoplasmic granules (i.e. neutrophils, eosinophils and basophils).
Granuloma	A tissue nodule containing proliferating lymphocytes, fibroblasts, and giant cells and epithelioid cells (both derived from activated macrophages), which forms due to inflammation in response to chronic infection or persistence of antigen in the tissues.

Granzymes	Serine esterases present in the granules of cytotoxic T lymphocytes and NK cells. They induce apoptosis in the target cell which they enter through perforin channels inserted into the target cell membrane by the cytotoxic lymphocyte.
Grb2	25 kDa Growth factor Receptor Binding protein-2.
Grk2	80 kDa G protein-coupled Receptor Kinase; serves to uncouple G protein- coupled receptors, which can lead to ERK activation.
GSK-3	46 & 54 kDa Glycogen Synthase Kinase; a serine/threonine kinase with two unique isoforms, Gsk-3 α and Gsk-3 β . GSK functions to phosphorylate glycogen synthase thereby inactivating it. Many other roles.
Gut-associated lymphoid tissue (GALT)	Includes Peyer's patches, appendix, and solitary lymphoid nodules in the submucosa.
H-2	The mouse major histocompatibility complex (MHC).
H-2 complex	The major histocompatibility complex situated on chromosome 17 of the mouse; contains subregions K, I and D.
Haemagglutinin	Any molecule that agglutinates erythrocytes.
Haematopoiesis	The production of erythrocytes and leukocytes.
Haploid	The number of chromosomes in a sperm or egg cell, half the diploid number.
Haplotype	A particular combination of closely linked genes on a chromosome inherited from one patient; the set of allelic variants present at a given genetic region.
Hapten	A compound, usually of low molecular weight, that is not itself immunogenic (but recognized by pre-formed antibody) that, after conjugation to a carrier protein or cells (which provides epitopes), becomes immunogenic and induces antibody, which can bind the hapten alone in the absence of carrier.
Hay fever	See Rhinitis.
Heavy chain (H chain)	The larger of the two types of chains that comprise a normal immunoglobulin or antibody molecule.
Helix	A secondary structural motif of proteins in which a linear sequence of amino acids folds into a right-handed helix stabilized by internal hydrogen bonding between backbone atoms.
Helper T cells	A subclass of T cells which help trigger B cells (in the form of cytokines and/or cognate interactions) to make antibody against thymus-dependent antigens. Helper T cells also help generate cytotoxic T cells.
Heterogeneous nuclear RNA (hnRNA)	RNA transcripts freshly synthesized by RNA polymerase II in the nucleus; also known as primary RNA transcript
Heterophile antigen	A cross-reacting antigen that appears in widely ranging species such as humans and bacteria.
Heterozygosity	The presence of different alleles at one or more loci on homologous chromosomes.
Heterozygous	Containing two different alleles of the same gene.
High endothelial venule (HEV)	Capillary venule composed of specialized endothelial cells allowing migration of lymphocytes into lymphoid organs.
Hinge region	Amino acids between the Fab and Fc regions of immunoglobulin which permit flexibility of the molecule. This area is susceptible to enzymatic cleavage.
Histocompatibility	Literally, the ability of tissues to get along; in immunology, it means identity in all transplantation antigens. These antigens, in turn, are collectively referred to as histocompatibility antigens.
Hit and run vector	Modified insertion vectors which have a mutation in the homologous sequences. These vectors will form a variety of different integration products upon targeting.
HLA	Human leukocyte antigen: The human major histocompatibility complex (MHC).
Homeobox	A short stretch of nucleotides whose base sequence is virtually identical in all the genes that contain it. It has been found in many organisms from fruit flies to human beings.
Homologous chromosomes	A pair of chromosomes containing the same linear gene sequences, each derived from one parent.
Homologous recombination	The exchange of DNA fragments between two DNA molecules or chromatids of paired chromosomes (during crossing over) at the site of identical nucleotide sequences.
Homozygote	An organism whose genotype is characterized by two identical alleles of a gene.
Homozygous	Containing 2 copies of the same allele.
Humanized antibody	A genetically engineered monoclonal antibody of non-human origin in which all but the antigen-binding CDR sequences have been replaced with sequences derived from human antibodies. This procedure is carried out to minimize the immunogenicity of therapeutic monoclonal antibodies.
Humoral	Pertaining to extracellular fluid such as plasma and lymph. The term humoral immunity is used to denote antibody-mediated immune responses.
Humoral immunity	Any immune reaction that can be transferred with immune serum is termed humoral immunity (as opposed to cell-mediated immunity). In general, this term refers to resistance that results from the presence of specific antibody.
Humoral response	The production of antibodies by plasma (B) cells; the defense by fluids ("humors") the antibody response.
Hybrid	An offspring of parents from different species or sub-species. Hybrid mice are generated by mating mice from two different inbred strains.
Hybridization	Process whereby two complementary nucleic acid strands form a double helix during an annealing period; a powerful technique for detecting specific nucleotide sequences.

Hybridoma	Hybrid cell line obtained by fusing a lymphoid tumor cell with a lymphocyte which then has both the immortality of the tumor cell and the effector function (e.g. monoclonal antibody secretion) of the lymphocyte. B cell hybridomas are usually used to make monoclonal antibodies.
Hydrogen bond	Bonds formed by polarized molecules (+ -), i.e. water.
Hydrolysis	Reaction in which water breaks the covalently linked compound A-B.
Hydrophilic	Water "loving," hydrophilic molecule are soluble in water.
Hydrophobic	Water "fearing," hydrophobic molecules are quite insoluble in water.
Hypersensitivity	State of reactivity to antigen that is greater than normal for the antigenic challenge, leading to undesirable consequences e.g. tissue or organ damage; hypersensitivity is the same as allergy and denotes a deleterious outcome rather than a protective one.
Hypervariable regions	Portions of the light and heavy immunoglobulin chains that are highly variable in amino acid sequence from one immunoglobulin molecule to another, and that, together, constitute the antigen-binding site of an antibody molecule. Also, portions of the T-cell receptor that constitute the antigen-binding site.
Ia	"Immune response-associated" proteins, found on B cells and antigen-presenting cells of mice; an old term now replaced with MHC (major histocompatibility complex) class II molecules.
Idiotope	An epitope made up of amino acids within the variable region of an antibody or T-cell receptor which reacts with an anti-idiotope.
Idiotypic	The combined antigenic determinants (idiotopes) found on antibodies or T cell receptor (in the variable region) of an individual that are directed at a particular antigen (and react with an anti-idiotypic serum); such antigenic determinants are found only in the variable region.
Idiotypic network	A regulatory network based on interactions of idiotypes and anti-idiotypes present on antibodies and T-cell receptors.
IkappaB alpha	38 kDa cytoplasmic inhibitory protein that exerts its effects on NFκB. Upon phosphorylation by IκB kinases, IκBα is degraded, allowing NFκB translocation to the nucleus where it regulates transcription. IκBα appears to be controlled by Bcl-2.
IkappaB beta	~38 kDa cytoplasmic inhibitory protein that exerts its effects on NFκB. Upon phosphorylation by IκB kinases, IκB is degraded allowing NFκB translocation to the nucleus where it regulates transcription. Phosphorylated by MEKK1.
IKK	IκB kinases that phosphorylate IκB. Kinase is present in two isoforms, IKKα and IKKβ.
IKKgamma (NEMO)	48 kDa noncatalytic component of the IκB kinase complex.
IL-2	Interleukin-2; a lymphokine required by activated T cells for growth.
IL-2R	Interleukin-2 receptor expressed on activated T and B lymphocytes.
IL-4	Interleukin-4; a cytokine produced by T cells to help antibody responses.
Immediate-type hypersensitivity	Hypersensitivity tissue reaction occurring within minutes after the interaction of antigen and antibody.
Immune adherence	The adherence of particulate antigen coated with C3b to tissue having cells with C3b receptors.
Immune complex	Complex of antibody bound to antigen which may also contain complement components.
Immune modulators	Substances that control the expression of the immune response.
Immune response (Ir) gene	A gene controlling an immune response to a particular antigen; most genes of this type are in the MHC (major histocompatibility complex), and the term is rarely used to describe other types of Ir genes outside the MHC.
Immune system	The immune system is a collection of cells and proteins that works to protect the body from potentially harmful, infectious microorganisms (microscopic life-forms), such as bacteria, viruses and fungi. The immune system plays a role in the control of cancer and other diseases, but also is the culprit in the phenomena of allergies, hypersensitivity and the rejection of transplanted organs, tissues and medical implants.
Immunity	Natural or acquired (specific) resistance to infection or disease.
Immunofluorescence	Technique for detection of cell or tissue-associated antigens by the use of a fluorescently-tagged ligand (e.g. an anti-immunoglobulin conjugated to fluorescein isothiocyanate).
Immunogen	A substance capable of inducing an immune response (as well as reacting with the products of an immune response). Whilst all immunogens are antigens, not all antigens are immunogens (<i>see</i> hapten).
Immunoglobulin superfamily	Large family of proteins characterized by possession of 'immunoglobulin-type' domains of approximately 110 amino acids folded into two β-pleated sheets. Members include immunoglobulins, T-cell receptors and MHC molecules.
Immunoglobulins	Immunoglobulins, also known as antibodies, are proteins found in blood and in tissue fluids. Immunoglobulins are produced by cells of the immune system called B-lymphocytes. Their function is to bind to substances in the body that are recognized as foreign antigens (often proteins on the surface of bacteria and viruses). 5 classes: IgG & IgM (humoral response), IgA (found in tears, saliva and milk), IgD (plays a role in B cell triggering) and IgE (histamine/inflammatory response). Each Ig unit is made up of two heavy chains and two light chains and has two antigen-binding sites.
Immunological synapse	A contact point between the T-cell and antigen-presenting cell which is generated by reorganization and clustering of cell surface molecules in lipid rafts. The synapse facilitates interactions between TCR and MHC and between adhesion molecules, thereby potentiating the TCR-mediated activation signal.

in situ	Refers to performing experiments or tests with intact tissues.
in situ hybridization	Use of a DNA or RNA probe to detect the presence of the complementary DNA sequence in cloned bacterial or cultured eukaryotic cells; is an effective method for analyzing transgene expression at the cellular level.
in vitro	A biologic or biochemical process occurring outside a living organism.
in vivo	A biologic or biochemical process occurring within a living organism.
Inbred strain	Strain of mice that has been maintained by successive brother to sister matings over more than 20 generations (i.e. BALB/c, C57BL/6, etc.).
Inducible ablation	Expression of the targeted gene is not by itself harmful to the cell but is capable of rendering cells selectively sensitive to the killing action of certain drugs that cannot be metabolized by normal cells. Example: expression of the herpes gene tk is not deleterious, however the addition of drugs to the cell extract (such as gancyclovir) kills dividing cells expressing the herpes tk gene. Hence expression of the herpes tk gene under the control of a cell type-specific promoter, followed by drug administration, constitutes an inducible system for achieving cell ablation.
Infectious tolerance	A self-perpetuating state of tolerance that can be transferred by T lymphocytes.
Inflammation	The tissue response to trauma, characterized by increased blood flow and entry of leukocytes into the tissues, resulting in swelling, redness, elevated temperature and pain.
Innate immunity	Immunity which is not intrinsically affected by prior contact with antigen, i.e. all aspects of immunity not directly mediated by lymphocytes.
Integrin receptor alpha/ beta	A 66 kDa receptor for integrins that are a group of alpha/beta heterodimeric proteins responsible for transducing intracellular signals on binding to the extracellular matrix.
Integrin-Associated Protein (IAP, CD47)	A 35-50 kDa multiple membrane spanning member of the immunoglobulin superfamily that regulates some adhesion-dependent cell functions through formation of a complex with alphaVbeta3 integrin and trimeric G proteins.
Interdigitating dendritic cell	MHC class II-positive, Fc receptor-negative, antigen-presenting dendritic cell found in T-cell areas of lymph nodes and spleen. (N.B. a different cell type to follicular dendritic cells).
Interferon	A substance produced by the body that inhibits viral DNA-RNA transcripts, i.e. group of cytokines that activate protection against viruses.
interferons (IFN)	IFN α is derived from various leukocytes, IFN β from fibroblasts and IFN γ from T lymphocytes. All three types induce an anti-viral state in cells and IFN γ acts as a cytokine in the regulation of immune responses.
Intrinsic asthma	Intrinsic asthma is asthma that has no apparent external cause.
Intron	A non-coding sequence of DNA that is initially copied into RNA but is cut out of the final RNA transcript; the intervening sequence of nucleotides between coding sequences or exons.
Invariant chain	A polypeptide which binds MHC class II molecules in the endoplasmic reticulum, directs them to the late endosomal compartment and prevents premature association with self peptides.
Ionic bond	Non-covalent bond; ionic interactions occur either between fully charged groups (ionic bond) or between partially charged groups.
Ir (immune response) genes	The genes, including those within the MHC, that together determine the overall level of immune response to a given antigen.
Isograft	A tissue transplanted between two genetically identical individuals.
Isohaemagglutinins	Antibodies to major red blood cell antigens present normally as a result of inapparent immunization by cross-reactive antigens in bacteria, food, etc.
Isotype switch	The shift of a B cell or its progeny from the secretion of antibody of one isotype or class of antibody with the same V regions but a different heavy-chain constant region and, hence, a different isotype (class switch).
Isotypes	Classes of antibody that differ in the constant region of their heavy chain (Fc portion); distinguishable also on the basis of reaction with antisera raised in another species. These differences also result in different biological activities of the antibodies.
ITAM	Immunoreceptor Tyrosine-based Activation Motifs are consensus sequences for src-family tyrosine kinases. These motifs are found in the cytoplasmic domains of several signaling molecules including the signal transduction units of lymphocyte antigen receptors and of Fc receptors.
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motifs present in the cytoplasmic domains of certain cell surface molecules, e.g. Fc γ RIIB, inhibitory NK cell receptors, and which mediate inhibitory signals.
J chain (joining chain)	A polypeptide involved in the polymerization of immunoglobulin molecules IgM (pentameric) and IgA (dimeric).
J gene	A gene segment coding for the J or joining segment in immunoglobulin DNA; V genes translocate to J segments in L chains, and to D and J segments in H chains. Also, codes for a portion of the T-cell receptor, and upon gene rearrangement, encode part of the third hypervariable region of the antigen receptors.
JAKs	Janus Activating Kinases; tyrosine kinases that are activated via tyrosine phosphorylation in response to cytokines and growth factors. Induction of JAK's (3 isotypes) lead to phosphorylation of STAT's.
JNKs (SAPKs)	c-Jun amino-terminal kinases (JNKs – 3 isoforms); a superfamily of Map kinase. Activated in response to stress stimuli, growth factors, and cytokines. JNK has been studied for its role in regulating cell fate, embryonic morphogenesis, cell proliferation, and apoptosis.

K (killer) cell	Large granular lymphocyte (express CD8), which mediates antibody-dependent cellular cytotoxicity (ADCC), and is Fc receptor positive, but does not rearrange or express either immunoglobulin or T-cell receptor genes. Also called cytotoxic T cells, and possesses a particular immune specificity.
Kinins	A family of polypeptides released during inflammatory responses and which increase vascular permeability and smooth muscle contraction.
KIRs	Killer cell Immunoglobulin-like Receptors found on NK cells, some $\alpha\beta$ and some $\gamma\delta$ T-cells. KIRs recognize MHC class I molecules and, like the C-type lectin receptors also found on these cells, can either inhibit or activate the killer cells. If ITIM sequences are present in their cytoplasmic domain they are inhibitory. KIRs lacking ITIMs can associate with ITAM-containing adaptor molecules, in which case they can activate the killer cell.
Knockout	The use of homologous genetic recombination in embryonal stem cells to replace a functional gene with a defective copy of the gene. The animals that are produced by this technique can be bred to homozygosity, thus allowing the generation of a null phenotype for that gene product.
Kupffer cells	Fixed tissue macrophages lining the blood sinuses in the liver.
Lagging strand	One of the two newly made strands of DNA found at the replication fork. The lagging strand is made in discontinuous lengths that are later joined covalently.
Langerhans' cell	Fc receptor and MHC class II-positive antigen-presenting dendritic cell found in the skin.
Large granular lymphocyte (LGL)	Large lymphocytes that contain cytoplasmic granules and function as natural killer (NK) and killer (K) cells. Activated CD8 cytotoxic T lymphocytes (Tc) also assume an LGL morphology.
LAT	40 kDa Linker for Activation of T cells; a palmitoylated integral membrane protein that is localized in the plasma membrane and promotes T cell activation by recruiting kinases to specific domains of the TCR complex.
Lck	A 56 kDa member of the Src non-receptor protein tyrosine kinase family that has an important role.
Leading strand	One of the two newly made strands of DNA found at the replication fork. The leading strand is made by continuous synthesis in the 5'-3' direction.
Lectins	A family of proteins, mostly of plant origin, which bind specific sugars on glycoproteins and glycolipids. Some lectins are mitogenic (e.g. PHA, ConA).
Leukotrienes	Metabolic products of arachidonic acid, which promote inflammatory processes (e.g. chemotaxis, increased vascular permeability) and are produced by a variety of cell types including mast cells, basophils and macrophages.
LFA-1	Lymphocyte associated antigen-1; a molecule involved in lymphocyte adhesion to antigen presenting cells.
Ligand	General term for a molecule recognized by a binding structure such as a receptor.
Light chain (L chain)	The light chain of immunoglobulin is a structural feature that occurs in two forms: kappa and lambda.
Limit of resolution	The limiting separation at which two objects can still be seen as distinct.
LIMK	LIM kinases are serine/threonine kinases that have 2 zinc finger motifs, known as LIM motifs, in their amino-terminal regulatory domain. LIMK are involved in the regulation of the actin cytoskeleton through Rho-family GTPases and their downstream kinases PAK and ROCK. ROCK/PAK phosphorylate LIMK in the activation loop, activating it. LIMK then inhibit the actin depolymerisation activity of cofilin.
Linkage	The proximity of two or more markers (e.g., genes, RFLP markers) on a chromosome; the closer together the markers are, the the probability that they will be separated during DNA repair or replication processes (binary fission in prokaryotes, mitosis or meiosis in eukaryotes), and hence the greater the probability that they will be inherited together.
Linkage disequilibrium	The occurrence at a greater frequency, in a population of linked genes, of two alleles being inherited together than that expected from the product of their individual frequencies, which is governed by factors other than change.
Linkage map	A map of the relative positions of genetic loci on a chromosome, determined on the basis of how often the loci are inherited together. Distance is measured in centimorgans (cM).
Lipid raft	Cholesterol- and glycosphingolipid-rich membrane subdomain in which molecules involved in cellular activation become concentrated.
Lipofection	The introduction of transgenes across cell membranes by using liposome vesicles formed by phagocytosis. This method is advantageous in that it is tissue-specific.
Lipopolysaccharide (LPS)	Endotoxin derived from Gram-negative bacterial cell walls, which has inflammatory and mitogenic actions.
Liposomes	Synthetic bilayer in the form of a spherical vesicle.
Locus	The position on a chromosome of a gene or other chromosome marker; also, the DNA at that position. The use of locus is sometimes restricted to mean regions of DNA that are expressed. See gene expression.
Lymph	The tissue fluid that drains into and through the lymphatic system.
Lymph nodes	Secondary lymphoid organs found throughout the body that contain mature lymphocytes capable of responding to foreign antigens.
Lymphadenopathy	Enlarged lymph nodes.
Lymphocyte	A lymphocyte is a group of white blood cells (with virtually no cytoplasm) of crucial importance to the adaptive part of the body's immune system, and includes the B and T cells. The adaptive portion of the immune system mounts a tailor-made defence when dangerous invading organisms penetrate the body's general defences. Found in blood, in all tissue, and in lymphoid organs, such as lymph nodes, spleen, and Peyer's patches, and bears antigen-specific receptors.

Lymphokine	Cytokine produced by lymphocytes.
Lymphokine-activated killer cells (LAK)	Killer (K) and natural killer (NK) cells activated <i>in vitro</i> by IL-2 to give enhanced killing of target cells.
Lymphotoxin (also called TNFα)	A T-cell derived cytokine which is cytotoxic for certain tumor cells and also has immunoregulatory functions.
Lyn	A 59 kDa member of the Src family proto-oncogene non-receptor protein tyrosine kinase family predominantly expressed in hematopoietic tissues.
Macromolecule	Molecule such as a protein, nucleic acid, or polysaccharide with a molecular mass greater than a few thousand Daltons.
Macrophage	A large phagocytic ("cell eating") white blood cell of the mononuclear series found within tissues. Properties include phagocytosis, and antigen presentation to T cells, and can mediate ADCC. Located in the tissues
Major histocompatibility complex (MHC)	A cluster of genes on chromosome 6 in humans, that are polymorphic cell surface proteins that characterize the tissue or cell as self or non-self (code for antigens which lead to rapid graft rejection between members of a single species which differ at these loci). These proteins also help to regulate the immune response of the T-cells. Several classes of protein such as MHC class I and II proteins are encoded in this region. These in humans, are known as 'Human leukocyte antigens' (HLA). See also 'MHC'.
Marginal zone	The outer area of the splenic periarteriolar lymphoid sheath (PALS) that is rich in B cells, particularly those responding to thymus-independent antigens.
Margination	Leukocyte adhesion to the endothelium of blood vessels in the early phase of an acute inflammatory reaction.
Marker	An identifiable physical location on a chromosome (e.g., restriction enzyme cutting site, gene) whose inheritance can be monitored. Markers can be expressed regions of DNA (genes) or some segment of DNA with no known coding function but whose pattern of inheritance can be determined. See RFLP, restriction fragment length polymorphism.
Mast cell	A tissue (particularly numerous in connective tissue, such as the dermis (innermost layer) of skin) cell with abundant granules which resembles the blood basophil. Both these cell types bear Fc receptors for IgE, which when crosslinked by IgE and antigen cause degranulation and the release of a number of mediators including histamine and leukotrienes. Participates in 'Immediate hypersensitivity' reactions.
Medulla	Inner (central) region of an organ.
MEF2c	51 kDa Transcription factor; regulates muscle-specific gene expression by binding to MAPK proteins.
Megakaryocyte	A bone marrow precursor of platelets.
MEK1/2	40-42 kDa Mitogen-activated ERK-activating Kinase, phosphorylates and activates MAPK/ERK family kinases; thereby regulating cell development and proliferation. Also known as MAPKK.
MEK3	A 71 kDa MEK protein that regulates JNK and p38.
MEK4	A 110 kDa MEK protein that regulates MAPK and JNK1.
MEKK1	A 161 kDa MEK kinase that regulates MEK activity by phosphorylation.
Membrane potential	Voltage difference across a membrane due to a slight excess of positive ions on one side and of negative ions on the other.
Membrane transport	Movement of molecules across a membrane mediated by a membrane transport protein.
Memory (immunological)	A characteristic of the acquired (adaptive) immune response of lymphocytes whereby a second encounter with a given antigen produces a secondary immune response; faster, greater and longer lasting than the primary immune response.
Memory cells	Clonally expanded T- and B-cells produced during a primary immune response and which are 'primed' to mediate a secondary immune response to the original antigen. After exposure to an antigen the first time the cells acquire a "molecular memory", so that on a second exposure to the same antigen they respond more quickly.
Messenger RNA (mRNA)	RNA that serves as a template for protein synthesis.
MHC class I molecule	A molecule encoded by genes of the MHC, which participates in antigen presentation to cytotoxic T (CD8+) cells. Class I MHC molecules are present on virtually all nucleated cells and are encoded mainly by the H-2K, D, and L loci in mice and by HLA-A, B, and C in man.
MHC class II molecule	A molecule encoded by genes of the MHC which participates in antigen presentation to helper T (CD4+) cells. Class II MHC molecules are expressed on antigen-presenting cells (primarily macrophages, B-cells and interdigitating dendritic cells) and are encoded by H-2A and E in mice and HLA-DR, DQ, and DP in man.
MHC restriction	The ability of T lymphocytes to respond only when they 'see' the appropriate antigen in association with "self" MHC class I or class II proteins (of the original haplotype associated with T-cell priming) on the antigen presenting cells.
Microvillus	Thin cylindrical membrane-covered projection on the surface of an animal cell containing a core bundled of actin filaments. They are present especially large numbers on the absorptive surface of intestinal epithelial cells.
Migration inhibition factor (MIF)	A lymphokine that inhibits the motility of macrophages in culture.

Minor histocompatibility antigens	These antigens (cell surface processed peptides), encoded outside the MHC (i.e. non-MHC encoded), are numerous, but do not generate rapid graft rejection or primary responses of T cells in vitro. They do not serve as restricting elements in cell interactions.
Mitochondria	Membrane-bounded organelle, about the size of bacterium, that carries out oxidative phosphorylation and produces most of the ATP in eucaryotic cells.
Mitogen	A substance that stimulates (non-specifically) the proliferation of many different clones of lymphocytes.
Mixed lymphocyte reaction (MLR)	When lymphocytes from two individuals are cultured together, a proliferative response is generally observed, as the result of reactions of T cells of one individual to MHC antigens (allogenic) on the other individual's cells.
MKP 1-6	43 kDa MAP Kinase PPase; dephosphorylates MAPK.
MLC2	Myosin is composed of six polypeptide chains: 2 identical heavy chains and 2 pairs of light chains. 'Myosin light chain 2' (MLC/LC/LC20) has many isoforms with different tissue distributions. MLC2 can be phosphorylated by MLCK and ROCK, and regulate actin:myosin contraction.
MLCK	129 kDa Myosin light chain kinase; phosphorylates myosin light chain protein.
MLCK PPase	115 kDa Myosin Light Chain Kinase PPase; dephosphorylates myosin light chain protein.
Monoclonal antibody	Homogeneous antibody derived from a single immortal B-cell clone (the progeny of a single cell) and therefore all bearing identical antigen-binding sites and isotype (single specificity).
Monocyte	Large circulating phagocytic white blood cell, 2-10% of total white cells, with indented nucleus. Migrates to tissues, where it is known as a macrophage.
Monokines	Soluble substances secreted by monocytes, which have a variety of effects on other cells.
Mononuclear phagocyte system	A system comprising blood monocytes and tissue macrophages.
mRNA	RNA molecule that specifies the amino acid sequence of a protein. See 'messenger RNA'.
MSK	A mitogen- and stress-activated kinase regulated by ERK and p38. Resembles p90 ^{RSK} .
mTOR	'Mammalian target of rapamycin' is a serine/threonine kinase regulated by PKB and activates p70S6K and inhibits the eIF4E inhibitor 4E-BP1.
Mucosal-associated lymphoid tissue (MALT)	Lymphoid tissue present in the surface mucosa of the respiratory, gastrointestinal and genitourinary tracts.
Multiple myeloma	Plasma cell malignancy resulting in high levels of monoclonal immunoglobulin in serum and of free light chains (Bence-Jones protein) in urine.
Murine	Pertaining to mice.
Mutation	Change in DNA sequence resulting from mutagens. Various types of mutations include frame-shift mutations, missense mutations, and nonsense mutations.
Myeloid cell	The lineages of bone-marrow-derived phagocytes (including neutrophils, eosinophils and monocytes), and other cells (mast cell, basophil, megakaryocyte).
Myeloma	A tumour of plasma cells, generally secreting a single species of immunoglobulin.
Myeloma protein	Monoclonal antibody secreted by myeloma cells.
Myosin	Made of light and heavy chains; regulates muscle contraction and action (530 kDa in size).
Naive	The state of lymphocytes before first exposure to their specific antigen.
Nck	An adaptor protein.
Negative selection	Deletion by apoptosis in the thymus of T-cells which recognize self peptides presented by self MHC molecules, thus preventing the development of autoimmune T-cells. Negative selection of developing B-cells is also thought to occur if they encounter high levels of self antigen in the bone marrow.
neoplasm (tumor)	A synonym for cancerous tissue.
Neutrophil	The major circulating phagocytic polymorphonuclear granulocyte. Enters tissues early in an inflammatory response and is also able to mediate antibody-dependent cellular cytotoxicity (ADCC).
NFAT-1	120 kDa Nuclear Factor of Activated T-cells; regulates expression of IL-2 in activated T-cells.
NFkappaB	65 + 105 kDa Nuclear Factor Kappa B, a transcription factor used as a primary regulator of a wide variety of proinflammatory mediators.
NIK	104 kDa NFkappaB Inducing Kinase.
NK (natural killer) cell	Large granular lymphocyte which does not rearrange nor express either immunoglobulin or T-cell receptor genes but is able to recognize and destroy certain tumor and virally-infected cells in an MHC and antibody-independent manner. Also, they participate in ADCC. They do not exhibit antigenic specificity, and their number does not increase by immunization.
NK-T cell	NK1.1 ⁺ lymphoid cells with a morphology and granule content intermediate between T-cells and NK cells. They are potent producers of IL-4, may be CD4 ⁺ 8 ⁻ or CD4 ⁺ 8 ⁺ , and express low levels of $\alpha\beta$ TCR with an invariant α chain and very restricted β chain specificity. Many of these TCR recognize antigens presented by the non-classical MHC-like molecule CD1. Their lectin-like NK1.1 receptor may recognize microbial carbohydrates.
Nonspecific response	A nonspecific response is immediate and general in nature; physical barriers such as the skin, chemicals such as lysozyme and the inflammatory response are involved in nonspecific responses.

Nucleolus	Structure in the nucleus where ribosomal RNA is transcribed and ribosomal subunits are assembled.
Nucleosides	Compound composed of a purine or pyrimidine base linked to either a ribose or deoxyribose sugar. DNA and RNA are not polymers of nucleosides.
Nucleotides	Serve as building blocks for the construction of nucleic acids. Nucleoside with one or more phosphate group joined in ester linkages to the sugar moiety. DNA and RNA are polymers of nucleotides.
Nucleus	Prominent membrane-bound organelle in a eucaryotic cell, containing DNA organized into chromosomes.
Null cells	An early population of lymphocytes bearing neither T-cell nor B-cell differentiation antigens.
Oedema	Swelling caused by accumulation of fluid in the tissues.
Okazaki fragments	Short lengths of DNA produced on the lagging strand during DNA replication.
Oncogene	A gene, one or more forms of which is associated with cancer. Many oncogenes are involved, directly or indirectly, in controlling the rate of cell growth.
Opsonin:	A substance (e.g. antibody or C3b) that coats a particle, such as a bacterium, and enhances phagocytosis by phagocytic cells (by promoting adhesion of the antigen to the phagocyte).
Opsonization	Literally means "preparation for eating". The coating of a bacterium (antigen) with antibody and/or complement (opsonin) that leads to enhanced phagocytosis of the bacterium by phagocytic cells.
Osmosis	Movement of solvent from low concentration of solute molecules to high concentration of solute molecules. The solvent moves from a hypotonic to a hypertonic solution. The two solutions that have identical solute concentrations are said to be isotonic.
Outbred strains	Strains of mice propagated by nonstandardized matings. These mice retain substantial genetic variability.
Oxidation	Removal of electrons.
Oxidative phosphorylation	The last step in catabolism and the point at which the major portion of metabolic energy is released.
p130cas	130 kDa Focal adhesion docking protein.
p190RhoGAP	190 kDa Rho GTPase activating protein; a multi-domain protein regulator of actin cytoskeleton.
p38 (ERK6) (MAPK/SAPK)	38 kDa MAPK family member required for growth in hyper- osmolar states.
p38d (SAPK4)	42 kDa MAPK family member that phosphorylates ATF-2 and MBP, but not MAPK APK-2 and -3.
p53	53 kDa Tumor suppressor gene.
p62 lck ligand/ZIP	62 kDa Cytoplasmic protein which binds to SH2 domain of lck.
p62dok	62 kDa GAP-associated protein which is tyrosine phosphorylated upon activation of the c-Kit receptor.
p90^{RSK}	A serine/threonine kinase activated by ERK1/2, PDK1 and PKC.
PAF (platelet activating factor)	An alkyl phospholipid released by a variety of cell types including mast cells and basophils, which has immuno- regulatory effects on lymphocytes and monocytes/macrophages as well as causing platelet aggregation and degranulation.
PAK	'p21-activated kinase' family is involved in multiple cellular processes, including cytoskeletal reorganization, MAPK signalling and apoptotic signalling. Rac/cdc42 binding to PBD domain causes autophosphorylation of PAK and a conformational change. PDK-1 phosphorylates and activates PAK. Has been implicated in cell motility, cell survival, cell growth, and actin reorganization.
Paracortex	The part of an organ (e.g. lymph node) that lies between the cortex and the medulla.
Paratope	An antibody-combining site that is complementary to an epitope.
Passive immunization	Immunization by the administration of preformed antibody into a nonimmune individual.
Passive transport (facilitated diffusion)	Movement of a molecule across a membrane down its concentration gradient.
pathogen-associated molecular pattern (PAMP)	Molecules such as lipopolysaccharide, peptidoglycan, lipoteichoic acids and mannans, which are widely expressed by microbial pathogens as repetitive motifs but are not present on host tissues. They are therefore utilized by the pattern recognition receptors (PRRs) of the immune system to distinguish pathogens from self-antigens.
pattern recognition receptor (PRR)	Receptors on professional antigen-presenting cells and phagocytes which enable them to recognize pathogen-associated molecular patterns (PAMPs). Amongst the large number of different PRRs are the mannose receptor (CD206) and the macrophage scavenger receptor (CD204).
Paxillin	68 kDa Cytoskeletal protein found at the end of actin stress fiber focal adhesions. It is a multidomain protein that is a key component of integrin signalling and required for cytoskeletal reorganization. Paxillin can be phosphorylated by FAK, and Crk can bind to Paxillin via its SH2 domain.
PDK-1	60-66 kDa Phosphoinositide-Dependent Kinase-1; A serine/threonine kinase that activates PKB, p70S6K, PKC and p90 ^{RSK} .
PECAM-1	82.5 kDa Platelet-Endothelial Cell Adhesion Molecule-1.
Peptide	A linear sequence of amino acids (often derived from proteins by degradation, but they can be artificially synthesised).
Perforin	Molecule produced by cytotoxic T-cells and NK cells which, like complement component C9, polymerizes to form a pore in the membrane of the target cell leading to cell death.
Periarteriolar lymphoid sheath (PALS)	The lymphoid tissue that forms the white pulp of the spleen.
Peroxisomes	Small membrane -bounded organelle that uses molecular oxygen to oxidize organic molecules.

Peyer's patches	Part of the gut associated lymphoid tissue (GALT) and found as distinct lymphoid nodules mainly in the small intestine.
PHA (phytohemagglutinin)	A plant lectin that acts as a T-cell mitogen.
Phage (bacteriophage)	Any virus that infects bacteria; widely used as cloning vectors.
Phage antibody library	A collection of cloned antibody variable region gene sequences which can be expressed as Fab or scFv fusion proteins with bacteriophage coat proteins. These can be displayed on the surface of the phages. The gene encoding a monoclonal recombinant antibody is enclosed in the phage particle and can be selected from the library by binding of the phage to specific antigen.
Phagocyte	Cells, including monocytes/macrophages and neutrophils, which are specialized for the engulfment of cellular and particulate matter.
Phagocytosis	The engulfment of a particle or a microorganism by leukocytes.
Phagolysosome	Intracellular vacuole where killing and digestion of phagocytosed material occurs following the fusion of a phagosome with a lysosome.
Phagosome	Intracellular vacuole produced following invagination of the cell membrane around phagocytosed material.
Phenotype	The physical expression (observable characteristics) of an individual's genotype.
Phorbol myristate acetate (PMA)	A mitogenic phorbol ester which directly stimulates protein kinase C and acts as a tumor promoter.
Phosphodiester linkage	Covalent bond that joins the 5' and 3' carbon atoms to form nucleic acids.
Phospholipid	The major category of lipid molecules used to construct biological membranes. Generally composed of two fatty acids linked through glycerol phosphate to one of a variety of polar groups.
Physical map	A map of the locations of identifiable landmarks on DNA (e.g., restriction enzyme cutting sites, genes), regardless of inheritance. Distance is measured in base pairs.
PI3K	Phosphatidylinositol-3-Kinase. Phosphorylates the D3 position of the inositol ring.
Pinocytosis	Ingestion of liquid or very small particles by vesicle formation in a cell.
PKA /cAMP dependent protein	40 kDa Protein Kinase A alpha isoform; a serine/threonine protein kinase activated by the second messenger cAMP.
PKB	See 'Akt'.
PKC alpha	81 kDa Protein Kinase C alpha isoform; a serine/threonine protein kinase involved in many cellular responses.
PKD	110/115 kDa Protein Kinase D; a serine/threonine kinase that can be activated in parallel or downstream of PKC. Also known as PKC μ .
PKR	65-68 kDa Protein Kinase R; a double-stranded RNA-activated serine/threonine protein kinase.
Plasma cell	Terminally differentiated B lymphocyte which actively secretes large amounts of antibody.
Plasma membrane	Encloses the cell, defines its boundaries, and maintains the essential differences between the cytosol and the extracellular environment
Plasmid	Autonomously replicating, extrachromosomal circular DNA molecules, distinct from the normal bacterial genome and nonessential for cell survival under nonselective conditions. Some plasmids are capable of integrating into the host genome. A number of artificially constructed plasmids are used as cloning vectors.
PLA2	An enzyme that catalyses the hydrolysis of glycerolipids to produce lysophospholipids and the release of arachidonic acid. Implicated in mitogenesis, differentiation, inflammation and cytotoxicity.
PLC	150 kDa Phospholipase C-gamma1. Breaks down PI(4,5)P2 lipids to form IP3 and DAG which serve as potent second messengers that activate multiple signaling cascades.
PLD	Phospholipase D hydrolyses phosphatidylcholine to produce choline and phosphatidic acid, which is the precursor of the second messenger DAG. Two isoforms identified and both regulated by protein kinases, small GTPases and calcium.
Pluripotent stem cells	Cells capable of giving rise to all classes of cells.
Pokeweed mitogen (PWM)	A plant lectin which is a T-cell dependent B-cell mitogen.
Poly-A tail	Addition made to RNA polymerase II's transcript; aids in the export of mature mRNA from the nucleus, affects the stability of at least some mRNAs in the cytoplasm, and seems to serve as a recognition signal for the ribosome that is required for efficient translation of mRNA.
Polyclonal	Many different clones, or the product of many different clones, e.g. polyclonal antiserum.
Polyclonal activator	A substance that induces activation of many individual clones of either T or B cells. See Mitogen.
Poly-Ig receptor	A receptor molecule which specifically binds J-chain containing polymeric Ig, i.e. dimeric secretory IgA and pentameric IgM, and transports it across mucosal epithelium.
Polymerase chain reaction (PCR)	A method for amplifying a DNA base sequence using a heat-stable polymerase and two 20-base primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Because the newly synthesized DNA strand can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce rapid and highly specific amplification of the desired sequence. PCR also can be used to detect the existence of the defined sequence in a DNA sample; technique for amplifying specific regions of DNA by multiple cycles of DNA polymerization, each followed by a brief heat treatment to separate complementary strands.

Polymorphism	Literally, "having many shapes"; in genetics polymorphism means occurring in more than one form within a species; the existence of multiple alleles at a particular genetic locus.
Polymorphonuclear leukocyte	White blood cell with granular cytoplasm, e.g. neutrophil
Polyribosomes (polysomes)	mRNA molecule to which are attached a number of ribosomes engaged in protein synthesis.
Positive selection	The selection of those developing T-cells in the thymus which are able to recognize self MHC molecules. This occurs by preventing apoptosis in these cells.
Primary immune response	The relatively weak immune response which occurs upon the first encounter of naive lymphocytes with a given antigen. The primary response is generally small, has a long induction phase or lag period, consists primarily of IgM antibodies, and generates immunologic memory.
Primary lymphoid organs	Organs in which the maturation of immunocompetent T and B lymphocytes take place and antigen-specific receptors are first acquired, i.e. bone marrow and thymus in mammals.
Prime	The process of giving an initial sensitization to antigen.
Probe	Single-stranded DNA or RNA molecules of specific base sequence, labeled either radioactively or immunologically, that are used to detect the complementary base sequence by hybridization.
Promoter	A site (nucleotide sequence) on DNA to which RNA polymerase will bind and initiate transcription.
Prophylaxis	Protection.
Prostaglandins	Acidic lipids derived from arachidonic acid which are able to increase vascular permeability, mediate fever, and can both stimulate and inhibit immunological responses.
Proteases	Enzymes, such as trypsin, that degrades proteins by hydrolyzing some of their peptide bonds.
Proteasome	Cytoplasmic proteolytic enzyme complex involved in antigen processing for association with MHC.
protein A	<i>Staphylococcus aureus</i> cell wall protein which binds to the Fc region of IgG.
Protein kinases	Enzymes that transfers the terminal phosphate group of ATP to a specific amino acid of a target protein.
Protein tyrosine kinases	Enzymes that are able to phosphorylate proteins on tyrosines, and often act in a cascade-like fashion in the signal transduction systems of cells.
Proteins	Long linear polymers of amino acids joined head to tail by peptide bond between carboxylic acid group of one amino acid to the amino group of the next.
Proteoglycans	Molecules consisting of one or more glucosaminoglycan (GAG) chains attached to a core protein.
PTEN	225 kDa MMAC1, a tumor suppressor gene; dephosphorylates D3-PI lipids and to some extent proteins.
Pyk2 (CAKbeta/ FAK2)	99-125 kDa Protein tyrosine Kinase, see CAKbeta.
Qa antigens	'Non-classical' MHC class I molecules of mice.
Rac/cdc42	21 kDa Members of the small molecular weight GTPase superfamily.
Radioallergosorbent test (RAST)	A solid-phase radioimmunoassay for detecting IgE antibody specific for a particular allergen (see 'RAST').
Radioimmunoassay (RIA)	A widely used technique for measurement of primary antigen-antibody interactions, and for the determination of the level of important biological substances in mixed samples. It takes advantage of the specificity of the antigen-antibody interaction and the sensitivity that derives from measurement of radioactively labelled materials.
Rac	A small GTPase
Raf1 (C-raf)	74 kDa Cytoplasmic Raf proto-oncogene serine/threonine protein kinase that is activated by Ras-GTP; a MAPKKK
RAFTK (CAKβ, Pyk2)	116 kDa Related Adhesion Focal Tyrosine Kinase. See CAKbeta, Pyk2.
Rap1-GAP (C3G)	73 kDa Inactivator of Rap-1.
Rap2	21 kDa Member of Ras family of low molecular weight GTP/GDP binding proteins; a small GTPase.
Ras	21 kDa GDP/GTP binding protein. Activates Raf-MEK-MAPK pathway; a small GTPase.
RAST	RAST is an abbreviation for RadioAllergoSorbent Test, a trademark of Pharmacia Diagnostics, which originated the test. RAST is a laboratory test used to detect IgE antibodies to specific allergens.
Receptor	Protein that binds to specific extracellular signaling molecule (ligand) and initiates a response in the cell. Cell-surface receptors such as the acetylcholine receptor and the insulin receptor, are located in the plasma membrane, with their ligand-binding site exposed to the external medium. Intracellular receptors, such as steroid hormonereceptors, bind ligands that diffuse into the cell across the plasma membrane.
Recessive	A genetic disorder that appears only in patients who have received two copies of a mutant gene, one from each parent.
Recombinant DNA	A combination of DNA molecules of different origin that are joined using recombinant DNA technologies.
Recombination	The process by which offspring derive a combination of genes different from that of either parent. In higher organisms, this can occur by crossing over.
Reduction	Addition of electrons.
Regulatory T-cell	A T lymphocyte that turns off specific immune responses.
Replication fork	Y-shaped region of replicating DNA molecule at which the two daughter strands are formed and separated.

Reporter gene	Encodes for an easily detectable protein. For example, lacZ, CAT (chloramphenicol acetyltransferase), and the luciferase genes all encode for bacterial enzymes. The presence of bacterial enzymes can be easily monitored by simple and sensitive assays of enzyme activity, without any interference from host cell enzymes. The regulatory sequence for the gene of interest can be identified by attaching the reporter sequence to various fragments of DNA sequence taken from upstream or downstream of the gene.
Respiratory burst	Oxygen dependent increase in metabolic activity (increased oxidative metabolism) within phagocytic cells stimulated by bacteria or parasites, to be microbicidal.
Respiratory chain	The name given to the electron-transport chain in the mitochondria
Restriction map	Diagrammatic representation of a DNA molecule indicating the sites of cleavage by various restriction enzymes.
Restriction nucleases	One of a large number of nucleases that can cleave a DNA molecule at any site where a specific short sequence of nucleotides occurs.
Reticuloendothelial system (RES)	An old term for the network of phagocytes and endothelial cells throughout the body.
Retroviral infection	Retroviral vectors with recombinant DNA incorporate their genome into the chromosomes of cells it infects.
Rheumatoid factor	An autoantibody (usually IgM, but also IgG and IgA) that reacts with the individual's own IgG, particularly the Fc region. Present in rheumatoid arthritis.
Rhinitis	Rhinitis is an inflammation of the mucous membrane that lines the nose, often due to an allergy to pollen, dust or other airborne substances. Seasonal allergic rhinitis also is known as "hay fever," a disorder that causes sneezing, itching, a runny nose and nasal congestion.
Rho	21.6 kDa Small molecular weight GTPase.
Ribosome	A complex composed of ribosomal RNAs and ribosomal proteins that associates with messenger RNA and catalyzes the synthesis of protein.
RIP	75-76 kDa Receptor Interacting Protein.
RNA polymerase	Enzyme that catalyzes the synthesis of an RNA molecule on a DNA template from nucleoside triphosphate precursors.
RNA polymerase I	Makes the large ribosomal RNAs.
RNA polymerase II	Transcribes genes whose RNA's will be translated into proteins.
RNA polymerase III	Makes a variety of very small, stable RNAs including 5s ribosomal RNAs and transfer RNAs.
RNA primers	Primers used to synthesize DNA strands by acting as a template.
RNA splicing	RNA-processing step in which all of the intron sequences are excised from RNA molecules in the nucleus, with exon sequences kept, thereby producing messenger RNA.
ROCK	160 kDa Rho-activated kinase.
rRNA	Any one of a number of specific RNA molecules that form part of the structure of a ribosome and participate in the synthesis of proteins.
S6k (p70S6K)	70 kDa S6 Kinase is a mitogen-activated serine/threonine kinase that plays a key role in the regulation of cell growth by controlling the biosynthesis of translational components, which make up the protein synthetic apparatus, most notably ribosomal proteins.
Second law of thermodynamics	The degree of disorder in the universe can only increase.
Secondary immune response	The qualitatively and quantitatively improved immune response which occurs upon the second encounter of primed lymphocytes with a given antigen.
Secondary lymphoid organs	Organs in which antigen-driven proliferation and differentiation of B and T lymphocytes takes place.
Secretory component	Proteolytic cleavage product of the poly-Ig receptor which remains associated with dimeric IgA in seromucus secretions.
Sex chromosome	One of the two chromosomes that specify an organism's genetic sex. Humans have two kinds of sex chromosomes, one called X and the other Y. Normal females possess two X chromosomes and normal males one X and one Y.
Sex-linked	Located on the X chromosome. Sex-linked (or X-linked) diseases are generally observed only in males.
Shc	Early signaling intermediates between the RTK and GPCR via Ras pathway; a scaffolding protein for a variety of RTKs. Exists in 3 forms: p46, p52 and p66 isoforms. Grb2/Sos binds to phosphorylated Shc, activating the Ras-Raf-MAPK pathway.
SHIP	145 kDa SH2 domain containing Inositol Phosphatase; dephosphorylates PIP3.
SHP1	68 kDa Protein Tyrosine Phosphatase which has 2 N-terminal SH2 domains.
SHP2	72 kDa Protein Tyrosine Phosphatase which has 2 N-terminal SH2 domains.
Side chain	Group attached to -carbon of amino acid and gives the amino acid its chemical properties.
Signalling molecules	Molecules involved in transmitting signals from the cell surface to the nucleus to switch genes on or off.
Single-stranded binding proteins (SSB)	Bind to exposed DNA strands without covering the bases; they aid helicase by stabilizing the unwound, single-stranded conformation.
Sinus	The sinuses (paranasal sinuses) are air cavities within the facial bones. They are lined by mucous membranes similar to those in other parts of the airways.

Sinusitis	Sinusitis is inflammation of the membrane lining the facial sinuses, often caused by bacterial or viral infection.
Slow-reacting substance of anaphylaxis (SRS-A)	A group of leukotrienes released by mast cells during anaphylaxis which induces a prolonged constriction of smooth muscle. This prolonged constriction is not reversible by treatment with antihistamines.
Small ribonucleoproteins (snRNPs)	Complexes of proteins with small RNAs contained in the nucleus; individually, they recognize specific nucleic acid sequences through RNA- RNA base-pairing.
Somatic cells	All body cells, except the reproductive cells.
Somatic hypermutation	The enhanced rate of point mutation in the immunoglobulin variable region genes which occurs following antigenic stimulation and acts as a mechanism for increasing antibody diversity and affinity.
SOS	153 kDa Son of Sevenless.
Southern blotting	Technique in which DNA fragments, separated by electrophoresis, are immobilized on a paper sheet; specific molecules are then detected with a labeled nucleic acid probe.
Spatial	Relating to, occupying, or having the character of space.
Specific immune response	An immune response to a specific antigen. This response can have a cell-mediated component and a humoral component (the production of antibodies).
Src	60 kDa pp60v-src, Rous Sarcoma Virus, a non-receptor tyrosine kinase that plays a role in ligand-induced cellular responses such as proliferation, survival, adhesion, and cell migration, as well as ion channel/receptor phosphorylation.
SRF	52 kDa Serum Response Factor.
Starch	Polysaccharide composed exclusively of glucose units, used as an energy store in plant cells.
STAT	'Signal transducers and activators of transcription' are regulated by JAKs, where upon phosphorylation they dimerise and translocate to the nucleus where they participate in transcriptional activation.
Stem cell	Multipotential cell from which differ-entiated cells derive.
Steroids	Hydrophobic molecules related to cholesterol. Many important hormones are steroids.
Superantigen	An antigen which reacts with all the T-cells belonging to a particular T-cell receptor V region family, and which therefore stimulates (or deletes) a much larger number of cells than does conventional antigen.
Suppression	A mechanism for producing a specific state of immunologic unresponsiveness by the induction of suppressor T cells. This type of unresponsiveness is passively transferable by suppressor T cells or their soluble products.
Suppressor T cells	Diminishes/controls specific immune response. Carries the CD8 antigen on their surface.
Syk	72 kDa Spleen tyrosine kinase (non-receptor). Plays critical role in signaling through immune receptors.
Synapses	Communicating cell-cell junction that allows signals to pass from a nerve cell to another cell.
Syndecans	22-46 kDa Family of four transmembrane proteoglycans; act as putative integrators of extracellular signals.
Syngeneic	Members of the same species that are genetically identical, e.g. a fully inbred strain of mice.
Syngraft	Same as isograft.
Synteny	Refers to genes on the same chromosome. Synteny conservation is defined as the occurrence of two or more pairs of homologous markers on the same chromosome in two or more species. Linkage conservation is conservation not only of synteny, but also of gene order.
T cell	A lymphocyte which undergoes a developmental stage in the thymus.
T cell receptor (TCR)	T cell antigen receptor that specifically recognizes processed antigens bound to MHC class I and II molecules. The TCR exists in two alternative forms, consisting of α and β chains, or γ and δ chains. The $\alpha\beta$ TCR recognizes peptide fragments of protein antigens presented by MHC molecules on cell surfaces. The function of the $\gamma\delta$ TCR is less clearly defined but it can recognize native proteins on the cell surface.
TAP	The Transporters associated with Antigen Processing (TAP-1 and TAP-2) are molecules which carry antigenic peptides from the cytoplasm into the lumen of the endoplasmic reticulum for incorporation into MHC class I molecules.
Targeted deletion	Technique for inactivating a gene by deleting it from the genome. May be accomplished by homologous recombination or inducible gene targeting.
Targeted mutagenesis	Alteration of the germline by the introduction of a site-directed mutation.
Targeting frequency	The frequency with which a vector transfected into cells recombines with the desired chromosomal target. The degree of homology in length and polymorphic variation between the vector and the host targeted sequence effect targeting frequency.
Tau	A 43 kDa Microtubule-associated protein involved in cytoskeleton regulation; when phosphorylated leads to formation of paired helical filaments observed in Alzheimer's disease and various tauopathies.
TCF	30-34 kDa T-cell Factor. Also known as Lymphoid Enhancer Factor.
T-dependent antigen	An antigen (immunogen) that requires helper T-cells in order to elicit an antibody response.
Temporal	Of or relating to time as distinguished from space; of or relating to the sequence of time or to a particular time : CHRONOLOGICAL .
Tensin	187 kDa Actin filament capping protein.
Th1	T lymphocytes making cytokines to help inflammation and anti-viral responses.
Th2	T lymphocytes making cytokines to help antibody responses.

Th3	T lymphocytes making predominantly TGF (and helping IgA antibody responses).
Thymocyte	Developing T-cell in the thymus.
Thymus	Primary lymphoid organ located above the heart from which T cells are derived.
Tiam 1	A guanine nucleotide-releasing factor for Rac.
T-independent antigen	An antigen (immunogen) that is able to elicit an antibody response in the absence of T-cells (lymphokines released by T cells). The antibodies are generally only of the IgM isotype.
Tissue type	Molecules on the surface of tissue cells recognised by the immune system.
T-lymphocyte	A nucleated white blood cell made in the thymus.
Tolerance	A non-aggressive state specific immunological tolerance) of the immune system normally associated with self-recognition.
Tolerogen	An antigen used to induce tolerance. Often depends more on the circumstances of administration (e.g. route and concentration) than on any inherent property of the molecule.
Tr1	T lymphocytes that regulate Th1 responses (may be related to Th3).
Transcription	The synthesis of an RNA copy from a sequence of DNA (a gene); the first step in gene expression.
Transfection	The uptake, incorporation, and expression of recombinant DNA by eukaryotic cells.
Transformation	Morphological changes in a lymphocyte associated with the onset of division. Also used to denote the change to the autonomously dividing state of a cancer cell.
Transforming growth factor (TGF)	A cytokine that down-regulates antigen presentation.
Transgene	The foreign gene.
Transgenic	This term describes an organism that has had genes from another organism put into its genome through recombinant DNA techniques. These animals are usually made by microinjection of DNA into the pronucleus of fertilized eggs, with the DNA integrating at random.
Transgenic line	A transgenic mouse strain in which the transgene is stably integrated into the germline and therefore inherited in Mendelian fashion by succeeding generations.
Transition state	Structure that forms transiently in the course of a chemical reaction and has the highest free energy of any reaction intermediate; a rate-limiting step in the reaction.
Translation	The process in which the genetic code carried by mRNA directs the synthesis of proteins from amino acids.
Transmembrane proteins	Amphipathic proteins that extend through the bilayer with part of their mass on both sides of the bilayer.
Triglyceride	Glycerol ester of fatty acids. The main constituent of fat droplets in animal tissues (where fatty acids are saturated) and of vegetable oil (where fatty acids are mainly unsaturated).
tRNA	Set of small RNA molecules used in protein synthesis as an interface (adapter) between mRNA and amino acids.
var der Waals attractions	At very short distances, any two atoms show a weak bonding interaction due to their fluctuating electrical charges. However, two atoms will very strongly repel each other if they are brought too close together. This latter phenomenon is known as van der Waals repulsions.
Variable (V) gene segments	Genes that rearrange together with <i>D</i> (diversity) and <i>J</i> (joining) gene segments in order to encode the variable region amino acid sequences of immunoglobulins and T-cell receptors.
Vasoactive amines	Substances including histamine and 5-hydroxytryptamine which increase vascular permeability and smooth muscle contraction.
Vav	The onc F proto-oncogene.
Vector	A DNA molecule that replicates on its own in a host cell and can be used as a vehicle in the laboratory for replicating other types of DNA.
Vinculin	117-130 kDa cytoskeletal protein which is found at the end of actin stress fiber focal adhesions.
Viruses	Obligate intracellular parasites that require the host cell's biochemical machinery to drive protein synthesis and metabolize sugars. Some produce acute infection and are eliminated from the host, whereas others persist indefinitely producing late disease.
Voltage-gated cation channels	Contained in the membrane of all electrically excitable cells; responsible for generating the action potentials.
V-Raf	68 kDa Viral serine/threonine protein kinase that is activated by Ras- GTP.
WASP	53 kDa Wiskott-Aldrich Syndrome Protein.
Western blot	A technique similar to Southern blotting, though it is used for proteins.
Xenogeneic	Genetic differences between species.
Xenograft	A tissue or organ graft between individuals of different species.
ZAP-70	A non-receptor tyrosine kinase.